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(57) Abstract

The invention discloses methods which are particularly sensitive for detecting low frequencies of mutations in mitochondrially encoded genes, such as the cytochrome b gene, making these an especially useful and commercially important way of screening plant pathogenic fungi for the onset of fungicidal resistance wherein the resistance is due to a mutation in a mitochondrially encoded gene. The methods disclosed include single nucleotide polymorphism detection techniques especially PCR detection methods. Also disclosed are DNA sequences encoding part of the wild type and mutant cytochrome b sequences of a range of plant pathogenic fungi and the use of the sequence information to detect mutations giving rise to fungicidal resistance. Allele specific oligonucleotides and oligonucleotide probes, diagnostic primers and diagnostic kits are also disclosed.

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METHODS

Field of the Invention

This invention relates to a diagnostic method for the detection of a cytochrome *b* mutation in fungi that leads to resistance to strobilurin analogues or compounds in the same cross resistance group using any (or a) single nucleotide polymorphism detection technique, preferably using the amplification refractory mutation system (ARMS). The invention also relates to mutation specific primers for use in the method and to diagnostic kits containing these primers. The invention further relates to the identification of a specific mutation in the fungal cytochrome *b* gene which results in the resistance of fungi containing the said mutation to strobilurin analogues or compounds in the same cross resistance group.

Background of the Invention

The widespread use of fungicides in agriculture is a relatively recent phenomenon, and most of the major developments have taken place during the last 40 years. Previously, farmers often ignored or did not recognise the effect that fungal pathogens had on the yield and quality of their crops. Nowadays, however, these losses are unacceptable, and farmers rely on the use of fungicidal chemicals to control fungal diseases. As a consequence, commercial fungicides have become an important component of the total agrochemical business, with world-wide sales in 1996 of about $5.9 billion, equivalent to 18.9% of the total agrochemical market (Wood Mackenzie, 1997a 'Agchem products- The key agrochemical product groups', in Agrochemical Service, Update of the Products Section, May 1997, 1-74). A large number of fungicides are already available to the farmer; a recent edition of The Pesticide Manual (Tomlin, 1994 10th Edition, British Crop Protection Council, Farnham, UK, and the Royal Society of Chemistry, Cambridge, UK) contains 158 different fungicidal active ingredients in current use. Nevertheless, further industrial research aimed at the discovery and development of new compounds is extremely intensive and product management procedures are extremely important in securing the best and longest lasting performance from fungicides with a particular mode of action and/or belonging to a particular compound series. In particular it is vital to develop effective resistance management strategies when fungicides with new modes of action are introduced (Fungicide Resistance Management : Into The Next Millenium (Russell) 1999, in Pesticide Outlook, October 1999 (213-215).

The strobilurin analogues constitute a major new series of agricultural fungicides which are considered the most exciting development on the agricultural fungicide scene since the discovery of the 1,2,4-triazoles in the 1970s.

The fungicidal activity of the strobilurin analogues is a result of their ability to inhibit mitochondrial respiration in fungi. More specifically, it has been established that these compounds have a novel single site mode of action, exerting their effect on fungi by blocking the ubiquinol:cytochrome c oxidoreductase complex (cytochrome *bc*1) thus reducing the generation of energy rich ATP in the fungal cell (Becker et al FEBS Letts. 132 329-33). This family of inhibitors prevents electron transfer at the ubiquinone redox site Q_o on the multimeric cytochrome *b* protein (Esposti et al 1993 Biochim. et Biophys Acta 243-271). Unlike many mitochondrial proteins, the cytochrome *b* protein is mitochondrially encoded.

Reports in the literature show that specific amino acid changes at the cytochrome *b* target site can affect the activity of strobilurin analogues. In depth mutagenesis studies in *Saccharomyces cerevisiae* (hereinafter referred to as *S. cerevisiae*) (JP Rago et al 1989 J. Biol. Chem. 264, 14543-14548), mouse (Howell et al 1988 J. Mol. Biol. 203, 607-618), *Chlamydomonas reinhardtii* (Bennoun et al 1991 Genetics 127, 335-343) and *Rhodobacter spp* (Daldal et al 1989 EMBO J. 3951-3961) have been carried out. Relevant information was also gathered from studying the natural basis for resistance to strobilurin analogues in the sea urchin *Paracentrotus lividus* (Esposti et al 1990 FEBS 263, 245-247) and the Basidiomycete fungi *Mycena galopoda* and *Strobilurus tenacellus* (Kraiczy et al 1996 Eur. J. Biochem. 235, 54-63), both of which produce natural variants of the strobilurin analogues. There are two distinct regions of the cytochrome *b* gene where amino acid changes have a dramatic effect on strobilurin analogue activity. These areas cover amino acid residues 125-148 and 250-295 (based on *S.cerevisiae* residue numbering system). More precisely amino acid changes at residues 126, 129, 132, 133, 137, 142, 143, 147, 148, 256, 275 and 295 have been shown to give rise to resistance to strobilurin analogues (Brasseur et al 1996 Biochim. Biophys. Acta 1275, 61-69 and Esposti et al (1993) Biochimica et Biophysica Acta, 1143, 243-271).

The present invention identifies for the first time the key importance of one of these mutations in cytochrome *b* gene of field isolates of important plant pathogenic fungi showing resistance to a strobilurin analogue or a compound in the same cross resistance group.

Summary of the Invention

According to a first aspect of the invention we provide a method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of said mutation in fungal nucleic acid using any (or a) single nucleotide polymorphism detection technique.

In the present invention we have now devised novel diagnostic methods for the detection of a point mutation in a fungal cytochrome *b* gene based on single nucleotide polymorphism detection methods including allele specific amplification. It will be apparent to the person skilled in the art that there are a large number of analytical procedures which may be used to detect the presence or absence of variant nucleotides at one or more polymorphic positions according to the invention. In general, the detection of allelic variation requires a mutation discrimination technique, optionally an amplification reaction and optionally a signal generation system. Many current methods for the detection of allelic variation are reviewed by Nollau et al, Clin. Chem. 43, 1114-1120, 1997 and in standard textbooks, for example 'Laboratory Protocols for Mutation Detection', Ed. bu U. Landegren, Oxford University Press, 1996 and 'PCR' 2nd Edition by Mewton and Graham, BIOS Scientific Publishers limited, 1997. Allele specific amplification reactions include primer based methods including PCR based methods and more specifically, allele specific polymerase chain reaction (PCR) extension (ASPCR) and specifically ARMS (Amplification Refractory Mutagenesis System) wherein the mutation gives rise to resistance to a strobilurin analogue and these are particularly preferred for use in the methods of the present invention. The methods of the invention also include indiscriminate PCRs followed by specific probing of the amplicon generated. These methods are suitable for the detection of the specific alleles which can confer resistance to any of the strobilurin analogues or any other compound in the same cross resistance group. Robust tests have been developed for the detection of this point mutation in a range of fungal plant pathogens. Compounds may be considered to be in the same cross resistance group when the resistance mechanism to one compound also confers resistance to another, even when the modes of action are not the same. The technique of ASPCR is described in US Patent No. 5639611 and the ARMS technique is described fully in European Patent No. EP 332435.

Other single polymorphism detection techniques which may be used to detect mutations include, for example, restriction fragment length polymorphism (RFLP), single

strand conformation polymorphism, multiple clonal analysis, allele-specific oligonucleotide hybridisation, single nucleotide primer extension (Juvonen et al, (1994) Hum Genet 93 16-20; Huoponen et al, (1994) Hum Mutat 3 29-36; Mashima et al (1995), Invest Opthelmol. Vision. Sci 36,1714-20; Howell et al (1994) Am J Hum Genet. 55 203-206; Koyabashi et al; (1994) Am. J. Hum. Genet. 55 206-209; Johns and Neufeld (1993) Am J Hum Genet 53 916-920; Chomyn et al, (1992) Proc. Natl. Acad. Sci USA 89 4221-4225) and Invader ™ technology (available from Third Wave Technologies Inc. 502 South Rosa Road,Madison, WI 53719 USA).

The use of PCR based detection systems is preferred.

According to a preferred embodiment of the first aspect of the invention we provide a method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid with a primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to presence or absence of said mutation in said nucleic acid.

The detection of the amplicon generated during the PCR reaction may be directly dependent on the extension of a primer specific for the presence of the mutation i.e. where primer extension is dependent on the presence of the mutation and hence an amplicon is generated only when the primer binds and/or is extended when the mutation is present (as is the case with ARMS technology), similarly it may be directly dependent on the extension of a primer specific for the absence of the mutation e.g. wild type sequence or may be directly linked to the PCR extension product containing the mutant DNA sequence i.e. where the detection is of an amplicon comprising the mutant DNA sequence. The first alternative is particularly preferred.

The amplicon can be from any PCR cycle and this includes a first allele specific primer extension product.

In a further preferred embodiment the invention provides a method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group which method comprises contacting a test sample comprising fungal nucleic acid with an

appropriate diagnostic primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended either when the said mutation is present in the sample or when wild type sequence is present; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

In a further preferred embodiment the invention provides a method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group which method comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the specific mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

According to a particularly preferred embodiment of the first aspect of the invention we provide a method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group which method comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the specific mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

As used herein the term diagnostic primer is used to indicate the primer which is used specifically to identify the presence or absence of a mutation or wild type sequence and the term common primer is used to denote a primer binding to the opposite strand of DNA to the diagnostic primer and 3' to the region recognised by that diagnostic primer and which, by acting with said diagnostic primer allows amplification of the intervening tract of DNA during the PCR. Where the diagnostic primer is an ARMS primer it can have a 3' mismatch when compared to the mutant or wild type sequence.

In this and all further aspects and embodiments of the invention it is preferred that the extension of the primer extension product is detected using a detection system which is an

integral part of either the diagnostic primer or the common primer on the opposite strand. This is described more fully herein.

The methods of the invention are particularly suitable for the detection of mutations in a mitochondrial gene which encodes a protein which is a target for a fungicide, more especially for the detection of mutations in a fungal cytochrome *b* gene where said mutations result in the inhibition of fungicide activity to the cytochrome b protein but still allow ATP generation to occur and most preferably wherein said mutation in the fungal cytochrome *b* gene results in one of the following amino acid substitutions: $A_{126}T$, $F_{129}L$, $Y_{132}C$, $C_{133}Y$, $G_{137}R/S/E/V$, $W_{142}T/K$, $G_{143}A$, $I_{147}F$, $T_{148}M$, $N_{256}Y/K/I$, $L_{275}F/S/T$ or $L_{293}F$ where the first amino acid is substituted by the second one at the position in the sequence denoted by the number, the presence of which give rise to fungal resistance to strobilurin analogues or any other compound in the same cross resistance group wherein the residue identification is based on the *S. cerevisiae* residue numbering system for cytochrome *b*.

The strobilurin analogues and compounds in the same cross resistance group include for example, azoxystrobin, picoxystrobin, kresoxim-methyl, trifloxystrobin, famoxadone and fenamidone.

We have found that the position in the fungal cytochrome *b* nucleic acid corresponding to the 143rd codon/amino acid in the cytochrome *b* of *S. cerevisiae* sequence is a key determinant of fungal resistance to strobilurin analogues or any other compound in the same cross resistance group in field isolates of strobilurin analogue resistant plant pathogenic fungi. The methods of the invention described herein are particularly suitable for the detection of a mutation at the position corresponding to *Saccharomyces cerevisiae* cytochrome *b* residue 143 where the glycine residue is replaced by another amino acid which inhibits the activity of strobilurin analogues or any other compound in the same cross resistance group and results in a resistant phenotype in the fungi carrying the mutant cytochrome *b* gene thereby giving rise to fungal resistance to strobilurin analogues or any other compound in the same cross resistance group.

The method is preferably used for the detection of a mutation resulting in the replacement of said glycine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 with an amino acid selected from the group arginine, serine, cysteine, valine, aspartic acid, glutamic acid, tryptophan and most preferably alanine.

In a yet further preferred embodiment of the first aspect of the invention we now provide a method for the detection of a mutation in a fungal cytochrome *b* gene resulting in a glycine to alanine replacement at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 ($G_{143}A$) in the encoded protein thereby giving rise to fungal resistance to strobilurin analogues or any other compound in the same cross resistance group said method comprising identifying the presence or absence of said mutation in fungal nucleic acid using any (or a) single nucleotide polymorphism detection technique.

The mutation in the fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein is usually a guanine to cytosine base change at the second position (base) of the codon and the detection of this single nucleotide polymorphism is preferred for all aspects and embodiments of the invention described herein.

In a still further preferred embodiment of the first aspect of the invention we now provide a diagnostic method for the detection of a mutation in a fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to presence or absence of said mutation in said nucleic acid.

In a particularly preferred embodiment of the first aspect of the invention we now provide a diagnostic method for the detection of a mutation in a fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein said method comprising contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the mutation resulting in a $G_{143}A$ replacement in the encoded protein in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when a mutation is present in the sample resulting in a $G_{143}A$ replacement in the encoded protein; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

In a further particularly preferred embodiment of the first aspect of the invention we now provide a diagnostic method for the detection of a mutation in a fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein said method comprising contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the

mutation resulting in a $G_{143}A$ replacement in the encoded protein in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when a mutation resulting in a $G_{143}A$ replacement in the encoded protein is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

As used herein the term $G_{143}A$ is used to denote the substitution of a glycine residue by an alanine residue in a fungal cytochrome *b* sequence at the equivalent of the position of the 143rd codon/amino acid of the *S. cerevisiae* cytochrome *b* sequence. This nomenclature is used for all the other residue changes quoted herein i.e. all positions are quoted relative to the *S. cerevisiae* cytochrome *b* protein sequence. The *S. cerevisiae* cytochrome *b* gene and protein sequences are available on the EMBL and SWISSPROT databases (See EMBL ACCESSION NO. X84042 and SWISSPROT ACCESSION NO. P00163). The skilled man will appreciate that the precise length and register of equivalent proteins from different species may vary as a result of amino or carboxy terminal and/or one or more internal deletions or insertions. Since the amino acid tract containing the residue corresponding to G_{143} in *S.cerevisiae* is well conserved (Widger *et al.* Proc.Nat.Acad.Sci., U.S.A. **81** (1984) 674-678) it is straightforward to identify the precisely corresponding residue in a newly obtained fungal cytochrome b sequence either by visual inspection or use of one of several sequence alignment programmes including Megalign or Macaw. Though designated G_{143} in this application, because of positional and functional equivalence, the precise position of this glycine in the new cytochrome *b* may not be the 143rd residue from its amino terminal end. The S. cerevisiae cytochrome b consensus sequence is provided in SWISSPROT ACCESSION NO. P00163. In all aspects and embodiments of the invention described herein the positions in the cytochrome *b* sequence are preferably as defined relative to the *S. cerevisiae* cytochrome *b* sequence provided in EMBL ACCESSION NO. X84042. Alternatively, in all aspects and embodiments of the invention described herein the positions in the cytochrome *b* sequence are preferably as defined relative to the *S. cerevisiae* cytochrome *b* consensus sequence as provided in SWISSPROT ACCESSION NO. P00163.

According to one aspect of the invention there is provided a method for the diagnosis of a single nucleotide polymorphism in a fungal cytochrome *b* gene which method comprises determining the sequence of fungal nucleic acid at a position corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *S.*

cerevisiae cytochrome *b* residue 143 in the cytochrome *b* protein and determining the resistance status of the said fungi to a strobilurin analogue or a compound in the same cross resistance group by reference to a polymorphism in the cytochrome *b* gene.

In all aspects and embodiments of the invention described herein it is preferred that only one base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein shows a mutation i.e there is a single nucleotide polymorphism occurring at one position only and it is further preferred that it is at the first or second base of the triplet and most especially is at the second base in the triplet.

According to a preferred embodiment of this aspect of the invention there is provided a method for the diagnosis of a single nucleotide polymorphism in a fungal cytochrome *b* gene which method comprises determining the sequence of fungal nucleic acid at a position corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein and determining the resistance status of the said fungi to a strobilurin analogue or a compound in the same cross resistance group by reference to a polymorphism in the cytochrome *b* gene.

In an embodiment of the above aspect of the invention the method for diagnosis described herein is one in which the single nucleotide polymorphism at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein is presence of G and/or C.

First position	Second position		Third position
5'end	C	G	3'end
G	Alanine	Glycine	U
	Alanine	Glycine	C
	Alanine	Glycine	A
	Alanine	Glycine	G

Table 1: codon usage

A glycine to alanine point mutation demands a G to a C change at the second base of the codon. Other mutations may also arise at the 3rd position in the codon due to degeneracy in genetic code for alanine and glycine (see Table 1) but this is readily taken into

consideration when designing the diagnostic primer. The diagnostic primer is preferably an ARMS primer. (The concept of ARMS primers is described fully in Newton et al, Nucleic Acid Research 17 (7) 2503-2516 1989). As a result ARMS primers can be designed for the detection of the $G_{143}A$ point mutation given only sequence information on the wild type, strobilurin analogue sensitive, cytochrome *b* gene. There is no need to have access to a resistant isolate in new fungi of interest resulting from a $G_{143}A$ mutation. Some examples of relevant plant pathogenic fungi are listed in Table 2. This list is not meant to be in any way to be exclusive. The skilled plant pathologist will be able to readily identify those fungi to which the methods of this invention are relevant.

	Examples of species in which $G_{143}A$ can be assayed :
1	*Plasmopara viticola*
2	*Erysiphe graminis f.sp. tritici/hordei*
3	*Rhynchosporium secalis*
4	*Pyrenophora teres*
5	*Mycosphaerella graminicola*
6	*Mycosphaerella fijiensis* var. *difformis*
7	*Sphaerotheca fuliginea*
8	*Uncinula necator*
9	*Colletotrichum graminicola*
10	*Pythium aphanidermatum*
11	*Colletotrichum gloeosporioides*
12	*Oidium lycopersicum*
13	*Leveillula taurica*
14	*Pseudoperonospora cubensis*
15	*Alternaria solani*
16	*Cercospora arachidola*
17	*Rhizoctonia solani*
18	*Venturia inaequalis*
19	*Magnaporthe grisea*
20	*Phytophthora infestans*

21	*Mycosphaerella musicola*

Table 2: Example of species where $G_{143}A$ can be assayed

The methods of the invention described herein are particularly useful in connection with plant pathogenic fungi and especially with the following fungal species: *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum glocosporioides, Oidium lycopersicum, Magnaporthe grisea, Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.*

In a further aspect the invention provides a method for detecting fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a single nucleotide polymorphism occurring at a position corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein.

In a further preferred embodiment of this aspect the invention provides a method for detecting fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a single nucleotide polymorphism occurring at a position corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein.

In a preferred embodiment of this aspect of the invention the presence or absence of a single nucleotide polymorphism at a position corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue

143 in the cytochrome *b* gene in fungal nucleic acid is identified using any (or a) single nucleotide polymorphism detection techniques .

The invention further provides a fungal DNA sequence encoding all or part of a wild type cytochrome *b* protein wherein said DNA sequence encodes a glycine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the wild type protein wherein said sequence is obtainable or obtained from a fungus selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctania solani, Mycosphaerella musicola and Cercospora arachidola.*

The fungal DNA sequences according to the above aspects of the invention preferably comprises around 30 nucleotides on either or both sides of the position in the DNA corresponding to one or more of the bases in the triplet, preferably corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein since this extent of nucleic acid provides the skilled man with all information necessary to design species and mutation specific reagents and/or methods for use in all single nucleotide polymorphism detection techniques. As used herein the term around 30 means that the sequence may comprise up to 30 nucleotides, for example 5, up to 10, 15, 20, or 25 nucleotides or may comprise more than 30 nucleotides.

As used herein in connection with all DNA and protein sequences the term 'all or part of' is used to denote a DNA sequence or protein sequence or a fragment thereof. A fragment of DNA or protein may for example be 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% of the length of the whole sequence.

It will be evident to the man skilled in the art that both samples containing genomic (mitochondrial) and cDNA may be analysed according to the invention. Where the sample contains genomic DNA intron organisation needs to be taken into account when using the sequence information. Examples of wild type fungal DNA sequences comprising part of the wild type cytochrome *b* gene sequence according to the above aspect of the invention are provided in the table below and said sequences form a further aspect of the invention.

Species	Sequence
Plasmopara viticola (cDNA & genomic)	5'TTTTGCCTTGGGGACAAATGAGTTTTTGGGGTGCAAC AGTTATTACAAATTTATTCTCGGC 3' (SEQ ID NO 1)
Erysiphe graminis f.sp. tritici/hordei cDNA & (cDNA & genomic)	5'TATTGCCATACGGGCAGATGAGCCACTGGGGTGCAAC CGTTATCACTAACCTAATGAGCGC 3' (SEQ ID NO 2)
Rhynchosporium secalis (cDNA & genomic)	5'TGCTTCCTTATGGACAGATGTCTTTATGAGGTGCCAC AGTTATAACTAATCTTATGAGTGC 3' (SEQ ID NO 3)
Pyrenophora teres (cDNA)	5'TTTTACCCTACGGGCAAATGAGCCTTTGAGGTGCTAC AGTTATTACTAACCTTATGAGTGC 3' (SEQ ID NO 4)
Pyrenophora teres (genomic)	5'TTTTACCCTACGGGCAAATGAGCCTTTGAGGTGAAAT ATTTGCCTCAAATGTATAACTAAT 3' (SEQ ID NO 5)
Mycosphaerella graminicola (cDNA & genomic)	5'TATTACCTTATGGTCAAATGTCTTTATGAGGAGCAAC AGTTATAACTAACTTATTGAGTGC 3' (SEQ ID NO 6)
Mycosphaerella fijiensis var. difformis (cDNA & genomic)	5'TTTTACCTTATGGTCAAATGTCTTTATGAGGAGCTACA GTTATAACTAATTTAATGAGCGC 3' (SEQ ID NO 7)
Sphaerotheca fuliginea (cDNA)	5'TACTTCCCTTCGGTCAAATGTCGCTCTGGGGTGCAAC CGTTATTACTAACCTTATGAGCGC 3' (SEQ ID NO 8)
Sphaerotheca fuliginea (genomic - *6bp upstream available)	5' *TCTGGGGTGCAACCGTTAAGTAATAGCGGTTGTAAAA (SEQ ID NO 9)
Uncinula necator (cDNA)	5'TTTTACCCTACGGGCAGATGAGCCTATGGGGTGCAAC CGTTATTACTAACCTTATGAGCGC 3' (SEQ ID NO 10)
Uncinula necator (genomic - *10bp upstream available)	5'*AGCCTATGGGGTGCAACCGTTAAGTAGGTAATAGCG GTTGA 3' (SEQ ID NO 11)
Colletotrichum graminicola (genomic & cDNA)	5'TTTTACCTTACGGACAAATGTCATTATGAGGTGCTAC AGTTATTACTAACCTTATAAGTGC 3' (SEQ ID NO 12)
Pythium aphanidermatum (genomic & cDNA)	5'TATTACCTTGGGGTCAAATGAGTTTTTGGGGTGCTAC TGTTATTACTAATTTATTTTCAGC 3' (SEQ ID NO 13)
Colletotrichum gloeosporioides (genomic & cDNA)	5'TTTTACCTTATGGACAAATGTCATTATGAGGTGCAAC AGTTATTACTAACCTTATAAGTGC 3' (SEQ ID NO 14)
Oidium lycopersicum (cDNA)	5'TTTTACCCTACGGGCAGATGAGCCTGTGGGGTGCAAC CGTTATTACTAACCTTATGAGCGC 3' (SEQ ID NO 15)
Leveillula taurica	5'TTTTACCATACGGACAAATGTCATTATGAGGTGCAAC

(cDNA)	AGTTATTACTAACCTTATGAGTGC 3' (SEQ ID NO 16)
Pseudoperonospora cubensis (cDNA & genomic)	5'TTTTACCTTGGGGACAAATGAGTTTTTGGGGTGCAAC TGTTATTACTAATTTATTTTCTGC 3' (SEQ ID NO 17)
Alternaria solani (cDNA & genomic)	5'TTCTTCCTTATGGGCAAATGTCTTTATGAGGTGCTACA GTTATTACTAACCTTATGAGTGC 3' (SEQ ID NO 18)
Cercospora arachidola (cDNA & genomic)	5'TATTACCTTATGGACAAATGTCATTATGAGGAGCTAC AGTTATTACTAATTTATTATCTGC 3' (SEQ ID NO 19)
Rhizoctonia solani (cDNA)	5'TGCTTCCATACGGGCAAATGTCTCTGTGGGGTGCTAC AGTAATTACTAATTTACTTTCTGC 3' (SEQ ID NO 20)
Mycosphaerella musicola (genomic & cDNA)	5'TTTTACCTTATGGTCAAATGTCTTTATGAGGAGCTACA GTTATAACTAATTTAATGAGTGC 3' (SEQ ID NO 21)

Table 3

In the above table the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 which results in the replacement of the normal glycine residue with an alternative amino acid wherein said replacement confers resistance to strobilurin analogues or a compound within the same cross resistance group is in bold and underlined.

The invention also extends to a fungal DNA sequence showing homology or sequence identity to said DNA sequences in Table 3 and covers for example, variations in DNA sequences found in different samples or isolates of the same species. These variations may, for example, be due to the use of alternative codon usage, varying intron/exon mitochondrial organisation and amino acid replacement.

In a further aspect the invention provides a fungal DNA sequence encoding all or part of a cytochrome *b* protein which, when said sequence is lined up against the corresponding wild type DNA sequence encoding a cytochrome *b* protein, is seen to contain a single nucleotide polymorphism mutation at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein which results in the replacement of the normal glycine residue with an alternative amino acid with the proviso that said DNA sequence is not the *Mycena galopoda* sequence encoding cytochrome *b*.

In a further preferred embodiment of this aspect the invention provides a fungal DNA sequence encoding all or part of a cytochrome *b* protein which, when said sequence is lined up against the corresponding wild type DNA sequence encoding a cytochrome *b* protein, is seen to contain a single nucleotide polymorphism mutation at a position in the DNA

corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein which results in the replacement of the normal glycine residue with an alternative amino acid with the proviso that said DNA sequence is not the *Mycena galopoda* sequence encoding cytochrome *b*.

The fungal DNA sequence according to the above aspect of the invention preferably comprises around 30 nucleotides on either or both sides of the position in the DNA corresponding to one or more of the bases in the triplet, preferably corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein since this extent of nucleic acid provides the skilled man with all information necessary to design species and mutation specific reagents and/or methods for use in all single nucleotide polymorphism techniques. As used herein the term around 30 means that the sequence may comprise up to 30 nucleotides, for example 5, up to 10, 15, 20, or 25 nucleotides or may comprise more than 30 nucleotides.

The invention further provides a fungal DNA sequence encoding all or part of a mutant cytochrome *b* protein wherein the presence of a mutation in said DNA confers resistance to a strobilurin analogue or a compound within the same cross resistance group, said mutation occurring at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein with the proviso that said DNA sequence is not the *Mycena galopoda* sequence encoding cytochrome *b*.

In a preferred embodiment of this aspect the invention further provides a fungal DNA sequence encoding all or part of a mutant cytochrome *b* protein wherein the presence of a mutation in said DNA confers resistance to a strobilurin analogue or a compound within the same cross resistance group, said mutation occurring at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein with the proviso that said DNA sequence is not the *Mycena galopoda* sequence encoding cytochrome *b*.

In the above aspects of the invention the mutation occurring at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein is preferably a guanine to cytosine base change.

The fungal DNA sequence encoding all or part of a mutant cytochrome *b* protein wherein the presence of a mutation in said DNA confers resistance to a strobilurin analogue or a compound within the same cross resistance group, according to the above aspects of the invention is preferably obtainable or obtained from a fungus selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea, Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.*

The invention extends also to DNA sequences comprising all or part of the sequences provided in Table 3 wherein the residue at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein is a cytosine residue (SEQ ID NOS 176 to 196). These sequences are shown below and form a further aspect of the invention:

5'TTTTGCCTTGGGGACAAATGAGTTTTTGGGCTGCAACAGTTATTACAAATTTATTCTCGGC 3' (SEQ ID NO 176).

5'TATTGCCATACGGGCAGATGAGCCACTGGGCTGCAACCGTTATCACTAACCTAA TGAGCGC 3'- (SEQ ID NO 177)

5'TGCTTCCTTATGGACAGATGTCTTTATGAGCTGCCACAGTTATAACTAATCTTAT GAGTGC 3'- (SEQ ID NO 178)

5'TTTTACCCTACGGGCAAATGAGCCTTTGAGCTGCTACAGTTATTACTAACCTTAT GAGTGC 3'- (SEQ ID NO 179)

5'TTTTACCCTACGGGCAAATGAGCCTTTGAGCTGAAATATTTGCCTCAAATGTATA ACTAAT 3'- (SEQ ID NO 180)

5'TATTACCTTATGGTCAAATGTCTTTATGAGCAGCAACAGTTATAACTAACTTATTGAGTG C 3' (SEQ ID NO 181)

5'TTTTACCTTATGGTCAAATGTCTTTATGAGCAGCTACAGTTATAACTAATTTAAT GAGCGC 3'- (SEQ ID NO 182)

5'TACTTCCCTTCGGTCAAATGTCGCTCTGGGCTGCAACCGTTATTACTAACCTTAT GAGCGC 3'- (SEQ ID NO 183)

5' *TCTGGGCTGCAACCGTTAAGTAATAGCGGTTGTAAAA- (SEQ ID NO 184)

5'TTTTACCCTACGGGCAGATGAGCCTATGGGCTGCAACCGTTATTACTAACCTTAT GAGCGC 3'- (SEQ ID NO 185)

5'*AGCCTATGGGCTGCAACCGTTAAGTAGGTAATAGCGGTTGA 3'- (SEQ ID NO 186)

5'TTTTACCTTACGGACAAATGTCATTATGAGCTGCTACAGTTATTACTAACCTTAT AAGTGC 3'- (SEQ ID NO 187)

5'TATTACCTTGGGGTCAAATGAGTTTTTGGGCTGCTACTGTTATTACTAATTTATT TTCAGC 3'- (SEQ ID NO 188)

5'TTTTACCTTATGGACAAATGTCATTATGAGCTGCAACAGTTATTACTAACCTTAT AAGTGC 3'- (SEQ ID NO 189)

5'TTTTACCCTACGGGCAGATGAGCCTGTGGGCTGCAACCGTTATTACTAACCTTAT GAGCGC 3'- (SEQ ID NO 190)

5'TTTTACCATACGGACAAATGTCATTATGAGCTGCAACAGTTATTACTAACCTTAT GAGTGC 3'- (SEQ ID NO 191)

5'TTTTACCTTGGGGACAAATGAGTTTTTGGGCTGCAACTGTTATTACTAATTTATT TTCTGC 3'- (SEQ ID NO 192)

5'TTCTTCCTTATGGGCAAATGTCTTTATGAGCTGCTACAGTTATTACTAACCTTAT GAGTGC 3'- (SEQ ID NO 193)

5'TATTACCTTATGGACAAATGTCATTATGAGCAGCTACAGTTATTACTAATTTATT ATCTGC 3'- (SEQ ID NO 194)

5'TGCTTCCATACGGGCAAATGTCTCTGTGGGCTGCTACAGTAATTACTAATTTACT TTCTGC 3'- (SEQ ID NO 195)

5'TTTTACCTTATGGTCAAATGTCTTTATGAGCAGCTACAGTTATAACTAATTTAATGAGTG C 3' (SEQ ID NO 196)

The invention also extends to a fungal DNA sequence showing homology or sequence identity to said DNA sequence containing said polymorphism and covers for example, variations in DNA sequences found in different samples of the same species. These variations may, for example, be due to the use of alternative codon usage, varying intron/exon mitochondrial organisation and amino acid replacement.

The DNA sequences encoding all or part of a wild type or mutant cytochrome *b* protein as described herein are preferably in isolated form. For example through being partially purified from any substance with which it occurs naturally. The DNA sequence is isolatable (obtainable) or isolated (obtained) from the fungi disclosed herein.

The invention further provides a computer readable medium having stored thereon any of the sequences described and claimed herein and including all or part of a DNA sequence or protein sequence encoding a mutant cytochrome *b* protein as herein described wherein the presence of a mutation gives rise to fungal resistance to a strobilurin analogue or any compound in the same cross resistance group; all or part of a DNA or protein sequence

encoding a mutant cytochrome *b* protein wherein said protein confers fungal resistance to a strobilurin analogue or a compound in the same cross resistance group from a fungus selected from the group *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum glocosporioides, Oidium lycopersicum, Magnaporthe grisea, Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola* and *Cercospora arachidola,* with the proviso that said DNA or protein sequence is not the Mycena galopoda cytochrome b sequence; all or part of a DNA or protein sequence encoding a wild type cytochrome *b* sequence from a fungus selected from the group *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum glocosporioides, Oidium lycopersicum, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola;* or any allele specific oligonucleotide; allele specific oligonucleotide probe, allele specific primer, common or diagnostic primer disclosed herein.

The computer readable medium may be used, for example, in homology searching, mapping, haplotyping, genotyping or any other bioinformatic analysis. Any computer readable medium may be used, for example, compact disk, tape, floppy disk, hard drive or computer chips.

The polynucleotide sequences of the invention, or parts thereof, particularly those relating to and identifying the single nucleotide polymorphisms identified herein, especially the G to C change in fungal cytochrome *b* causing the $G_{143}A$ change in the encoded protein, represent a valuable information source. The use of this information source is most easily facilitated by storing the sequence information in a computer readable medium and then using the information in standard bioinformatics programs. The polynucleotide sequences of the invention are particularly useful as components in databases for sequence identity and other search analyses. As used herein, storage of the sequence information in a computer readable medium and use in sequence databases in relation to polynucleotide or polynucleotide sequence of the invention covers any detectable chemical or physical

characteristic of a polynucleotide of the invention that may be reduced to, converted into or stored in a tangible medium, such as a computer disk, preferably in a computer readable form. For example, chromatographic scan data or peak data, photographic scan or peak data, mass spectrographic data, sequence gel (or other) data.

A computer based method is also provided for performing sequence identification, said method comprising the steps of providing a polynucleotide sequence comprising a polymorphism of the invention in a computer readable medium and comparing said polymorphism containing polynucleotide sequence to at least one other polynucleotide or polypeptide sequence to identify identity (homology) i.e. screen for the presence of the polymorphism.

The invention further provides a fungal cytochrome *b* protein which confers fungal resistance to a strobilurin analogue or a compound within the same cross resistance group wherein in said protein a normal glycine residue is altered due to the presence of a mutation in the DNA coding for said protein said mutation occurring at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein with the proviso that said sequence is not the *Mycena galopoda* cytochrome *b* sequence.

In a preferred embodiment of this aspect the invention further provides a fungal cytochrome *b* protein which confers fungal resistance to a strobilurin analogue or a compound within the same cross resistance group wherein in said protein a normal glycine residue is altered due to the presence of a mutation in the DNA coding for said protein said mutation occurring at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein with the proviso that said sequence is not the *Mycena galopoda* cytochrome *b* sequence.

The *Mycena galopoda* cytochrome *b* sequence is described by Kraiczy et al (Eur. J. Biochem. 235, 54-63 (1996)) and the DNA sequence is in the EMBL data base EMBL Acession No. X87997.

The glycine residue in the protein according to the above aspect of the invention is preferably replaced by an alternative amino acid and said replacement results in the said fungi showing resistance to a strobilurin analogue or any other compound in the same cross resistance group.

The mutation according to the above aspect of the invention preferably results in the replacement of said glycine residue with an amino acid selected from the group arginine, serine, cysteine, valine, aspartic acid, glutamic acid and most preferably alanine.

In a further aspect the invention provides an antibody capable of recognising said mutant cytochrome *b* protein.

In a further aspect the invention provides a method for the detection of a mutation in fungal cytochrome *b* gene resulting in replacement in the encoded protein of a glycine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 said method comprising identifying the presence and absence of said mutation in a sample of fungal nucleic acid wherein any (or a) single nucleotide polymorphism detection method is based on the sequence information from around 30 to 90 nucleotides upstream and/or downstream of the position corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in either the wild type or mutant protein.

In a further preferred embodiment of this aspect the invention provides a method for the detection of a mutation in fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein said method comprising identifying the presence and absence of said mutation in a sample of fungal nucleic acid wherein any (or a) single nucleotide polymorphism detection method is based on the sequence information from around 30 to 90 nucleotides upstream and/or downstream of the position corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in either the wild type or mutant protein.

In a further preferred embodiment of this aspect the invention provides a method for the detection of a guanine to cytosine mutation in a fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein said method comprising identifying the presence and absence of said mutation in a sample of fungal nucleic acid wherein any (or a) single nucleotide polymorphism detection method is based on the sequence information from around 30 to 90 nucleotides upstream and/or downstream of the position corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in either the wild type or mutant protein.

The sequence information according to the above aspect of the invention is preferably derived from a fungus selected from the group *Plasmopara viticola, Erysiphe graminis f.sp.*

tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporiondes, Oidium lycopersicum, Magnaporthe grisea, Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola* and *Cercospora arachidola* and more preferably from the group *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctania solani, Mycosphaerella musicola and Cercospora arachidola.*

As used herein the term around 30 means that the sequence may comprise up to 30 nucleotides, for example 5, up to 10, 15, 20, or 25 nucleotides or may comprise more than 30 nucleotides. In the above aspects of the invention it is preferred that the sequence information used is around 30, preferably 30 nucleotides upstream and/or downstream of the position corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in either the wild type or mutant protein.

The nucleic acid according to the invention is preferably DNA, the test sample of nucleic acid is conveniently a total DNA preparation from fungal material, a cDNA preparation from fungal material or the fungal material itself or plant or seed extracts containing fungal nucleic acid. In this invention, we describe the detection of the $G_{143}A$ mutation by using total DNA preparation, cDNA preparation and by directly using fungal spore material as template in the PCRs. It will be appreciated that the test sample may equally be a nucleic acid sequence corresponding to the sequence in the test sample. That is to say that all or a part of the region in the sample nucleic acid may firstly be isolated or amplified using any convenient technique such as PCR before use in the method of the invention.

The present invention provides a means of analysing mutations in the DNA of agricultural field samples which by their very origin are considerably less well defined compared with an analogous situation involving human samples. Agricultural field samples

are considerably more difficult to work with and it is more technically demanding to detect a mutation event occurring at a low frequency in amongst a very large amount of wild type DNA and/or extraneous DNA from other organisms present in a field isolate compared with a human sample which generally contains DNA from only one individual.

Any convenient enzyme for polymerisation may be used provided that it has no intrinsic ability to discriminate between normal and mutant template sequences to any significant extent. Examples of convenient enzymes include thermostable enzymes which have no significant 3'-5' exonuclease activity, for example Taq DNA polymerase, particularly 'Ampli Taq Gold' ™ DNA polymerase (PE Applied Biosystems), Stoffel fragment, or other appropriately N-terminal deleted modifications of Taq (*Thermus aquaticus*) or Tth (*Thermus thermophilus*) DNA polymerases.

In a further aspect the invention provides an allele specific oligonucleotide capable of binding to all or part of a fungal nucleic acid sequence encoding a wild type cytochrome *b* protein selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum glocosporioides, Oidium lycopersicum, Magnaporthe grise, Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola* wherein said oligonucleotide comprises a sequence which recognises a nucleic acid sequence encoding a glycine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143.

In a preferred embodiment of this aspect of the invention the said fungal nucleic acid sequence is selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum glocosporioides, Oidium lycopersicum, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.*

The fungal nucleic acid according to the above aspects of the invention is preferably DNA.

In a further aspect of the invention we provide an allele specific oligonucleotide capable of binding to a fungal nucleic acid sequence encoding all or part of a mutant cytochrome *b* protein wherein said oligonucleotide comprises a sequence which recognises a nucelic acid sequence encoding an amino acid selected from the group arginine, serine, cysteine, valine, aspartic acid, glutamic acid , tryptophan, and most preferably alanine at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143.

In a preferred embodiment of this aspect of the invention we provide an allele specific oligonucleotide capable of binding to a fungal nucleic acid sequence encoding all or part of a mutant cytochrome *b* protein selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea, Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola* wherein said oligonucleotide comprises a sequence which recognises a nucleic acid sequence encoding an amino acid selected from the group arginine, serine, cysteine, valine, aspartic acid, glutamic acid, tryptophan, and most preferably alanine at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143.

The fungal nucleic acid according to the above aspects of the invention is preferably DNA.

In a further aspect the invention provides an allele specific oligonucleotide probe capable of detecting a fungal cytochrome *b* gene polymorphism at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein.

In a further preferred embodiment of this aspect the invention provides an allele specific oligonucleotide probe capable of detecting a fungal cytochrome *b* gene polymorphism at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein.

In further preferred embodiments of the above aspect of the invention said polymorphism is a guanine to cytosine base change, the mutation is in a fungus selected from

the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea, Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.*

The allele-specific oligonucleotide probe is preferably 17 to 50 nucleotides long, more preferable about 17-35 nucleotides long and most preferable about 17-30 nucleotides long.

The design of such probes will be apparent to the molecular biologist of ordinary skill and may be based on DNA or RNA sequence information. Such probes are of any convenient length such as up to 50 bases, up to 40 bases, more conveniently up to 30 bases in length, such as for example 8-25 or 8-15 bases in length. In general such probes will comprise base sequences entirely complementary to the corresponding wild type or variant locus in the gene. However, if required one or more mismatches may be introduced, provided that the discriminatory power of the oligonucleotide probe is not unduly affected. The probes of the invention may carry one or more labels to facilitate detection.

The invention further provides nucleotide primers which can detect the nucleotide polymorphisms according to the invention.

According to another aspect of the invention there is provided an allele specific primer capable of detecting a cytochrome *b* gene polymorphism at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein.

According to a preferred embodiment of this aspect of the invention there is provided an allele specific primer capable of detecting a cytochrome *b* gene polymorphism at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein.

In the above aspects the said mutation in the DNA sequence is preferably a guanine to cytosine base change.

In a further aspect the invention provides an allele specific primer capable of detecting a fungal DNA sequence encoding a wild type cytochrome *b* protein selected from

the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea, Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola* wherein said primer is capable of detecting a DNA sequence encoding a glycine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143.

In a preferred embodiment of this aspect of the invention the said fungal DNA sequence is selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.*

In a further aspect of the invention we provide an allele specific primer capable of detecting a fungal DNA sequence encoding all or part of a mutant cytochrome *b* protein wherein said allele specific primer is capable of detecting a DNA sequence encoding an amino acid selected from the group arginine, serine, cysteine, valine, aspartic acid, glutamic acid, tryptophan, and most preferably alanine at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143.

In a preferred embodiment of this aspect of the invention we provide an allele specific primer capable of detecting a fungal DNA sequence encoding all or part of a mutant cytochrome *b* protein selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea, Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola* wherein said primer is capable of detecting a DNA sequence encoding an amino acid selected from the group arginine,

serine, cysteine, valine, aspartic acid, glutamic acid, tryptophan, and most preferably alanine at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143.

An allele specific primer is used generally with a common primer in an amplification reaction such as a PCR reaction which provides the discrimination between alleles through selective amplification of one allele at a particular sequence position e.g as used in the ARMS assay.

We have now devised primers for the $G_{143}A$ mutation in the above-listed fungal species which have been shown to detect the specific mutations reliably and robustly. The primers detect the guanine to cytosine base change at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein . The allele specific primers are herein referred to as diagnostic primers. In a further aspect the invention therefore provides a diagnostic primer capable of binding to a template comprising a mutant type fungal cytochrome *b* nucleotide sequence wherein the final 3` nucleotide of the primer corresponds to a nucleotide present in said mutant form of a fungal cytochrome *b* gene and the presence of said nucleotide gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group.

The diagnostic primer of the invention is preferably at least 20 nucleotides in length, most preferably 26 nucleotides in length but this may be between 15 and 20 nucleotides in length.

In a preferred embodiment of the above aspect of the invention the penultimate nucleotide (-2) of the primer is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence.

In a further preferred embodiment it is the -3 nucleotide of the primer which is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence.

Other destabilising components may be incorporated along with the -2 or -3 nucleotide.

In a further particularly preferred embodiment of the above aspect of the invention we provide diagnostic primers capable of binding to a template comprising a mutant type fungal cytochrome *b* nucleotide sequence wherein the final 3` nucleotide of the primer corresponds to a nucleotide present in said mutant form of a fungal cytochrome *b* gene and wherein up to

10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides may be varied with respect to the wild type sequence without significantly affecting the properties of the diagnostic primer.

In a further particularly preferred embodiment of the above aspect of the invention we provide diagnostic primers comprising the sequences given below and derivatives thereof wherein the final nucleotide at the 3' end is identical to the sequences given below and wherein up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides may be varied without significantly affecting the properties of the diagnostic primer.

Conveniently, the sequence of the diagnostic primer is exactly as provided below. It is preferred that the ARMS primers in all aspects of the invention are 26 nucleotides in length. In the majority of the primers listed below the penultimate nucleotide has been altered from wild type cyt *b* sequence to destabilise the primer thereby making it more selective for the desired template and these primers are particularly preferred according to the invention. It will be apparent to the man skilled in the art of primer design that bases alternative to or in addition to those discussed above may also be varied without adversely affecting the ability of the primer to bind to the template.

Primer #	species:	primer sequence for the detection of $G_{143}A$ (5' to 3')
1	*Plasmopara viticola*	CCTTGGTGACAAATGAGTTTTTGGAC (SEQ ID NO 22)
2	*Erysiphe graminis f.sp. tritici/hordei*	CCATACGGGCAGATGAGCCACTGGAC (SEQ ID NO 23)
3	*Rhynchosporium secalis*	CCTTATGGACAGATGTCTTTATGATC (SEQ ID NO 24)
4	*Pyrenophora teres*	CCCTACGGGCAAATGAGCCTTTGCGC (SEQ ID NO 25)
5	*Mycosphaerella graminicola*	CCTTATGGTCAAATGTCTTTATGAAC (SEQ ID NO 26)
6	*Mycosphaerella fijiensis* var. *difformis*	CCTTATGGTCAAATGTCTTTATGATC (SEQ ID NO 27)
7	*Sphaerotheca fuliginea*	CCCTTCGGTCAAATGTCGCTCTGGAC (SEQ ID NO 28)
8	*Uncinula necator*	CCCTACGGGCAGATGAGCCTATGGTC (SEQ ID NO 29)
9	*Colletotrichum graminicola*	CCTTACGGACAAATGTCATTATGAAC

		(SEQ ID NO 30)
10	*Pythium aphanidermatum*	CCTTGGTGTCAAATGAGTTTTTGGAC (SEQ ID NO 31)
11	*Colletotricium gloeosporioides*	CCTTATGGACAAATGTCATTATGAAC (SEQ ID NO 32)
12	*Oidium lycopersicum*	CCCTACGGGCAGATGAGCCTGTGGAC (SEQ ID NO 33)
13	*Leveillula taurica*	CCATACGGACAAATGTCATTATGAAC (SEQ ID NO 34)
14	*Pseudoperonospora cubensis*	CCTTGGGGACAAATGAGTTTTTGGAC (SEQ ID NO 35)
15	*Alternaria solani*	CCTTATGGGCAAATGTCTTTATGAAC (SEQ ID NO 36)
16	*Cercospora arachidola*	CCTTATGGACAAATGTCATTATGAAC (SEQ ID NO 37)
17	*Rhizoctonia solani*	CCATACGGGCAAATGTCTCTGTGGAC (SEQ ID NO 38)
18	*Venturia inaequalis*	GTGTATGGTCAAATGAGCCTATGGCC (SEQ ID NO 39)
19	*Magnaporthe grisea*	CCTTATGGACAGATGTCATTATGAAC (SEQ ID NO 40)
20	*Phytophthora infestans*	CCTTGGGGACAAATGAGTTTTTGGAC (SEQ ID NO 41)
21	*Mycosphaerella musicola*	CCTTATGGTCAAATGTCTTTATGATC (SEQ ID NO 42)

Table 4: ARMS primer design for the detection of the $G_{143}A$ mutation

For the purposes of exemplification the primers included in Table 4 include:

- ARMS primers for *P.teres, and V. ineaqualis* which can be used effectively either on genomic DNA preparations or biological samples including fungal isolates, fungal cultures, fungal spores or infected plant material.
- ARMS primers for *S. fulgmea, O. lycopersicon, L. taurica, U. necator, Phytopthora infestans, Magnoporthe grisea, R.solani* which may only be effective on cDNA
- ARMS primers for *P.viticola, E.graminis f.sp tritici or hordei, R. secalis, M. graminicola, M. fijiensis var.difformis, C. graminicola, P. aphanidermatum, C. gloesporoides, P. cubensis, C. arachidola, Mycosphaerella musicola* and *A. solani* which may be used effectively with either genomic DNA preparations, cDNA preparations, cDNA

preparations or biological samples incuding fungal isolates, fungal cultures, fungal spores or infected plant material.

cDNA material is recommended for the species where the intron/exon organisation is not currently characterised around the single nucleotide polymorphism (SNP) of interest.

To adapt the primers shown in the above Table for use in a standard ASPCR reaction the last base at the 3` end should correspond to the point mutation without a destabilising base introduced.

Such primers may be manufactured using any convenient method of synthesis. Examples of such methods may be found in standard textbooks, for example "Protocols For Oligonucleotides And Analogues: Synthesis And Properties;" Methods In Molecular Biology Series: Volume 20: Ed. Sudhir Agrawal, Humana ISBN: 0-89603-247-7; 1993; 1st Edition.

It will be appreciated that extension of a diagnostic primer can be designed to indicate the absence of the mutation resulting in a $G_{143}A$ replacement in the encoded protein. The use of ARMS primers for the detection of the absence of the mutation resulting in a $G_{143}A$ replacement in the encoded protein is preferred. Primers designed for that purpose are described herein.

The detection of the wild type sequence is useful as a control in relation to the detection of the mutation and also is necessary where quantitation of wild type and mutant alleles present in a sample is desired.

In a further aspect the invention therefore provides a diagnostic primer capable of binding to a template comprising wild type fungal cytochrome *b* nucleotide sequence wherein the final 3` nucleotide of the primer corresponds to a nucleotide present in a wild type fungal cytochrome *b* gene said wild type fungus showing sensitivity to a strobilurin analogue or any other compound in the same cross resistance group.

In a preferred embodiment of this aspect of the invention the penultimate nucleotide (-2) of the primer is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence.

In a further preferred embodiment the -3 nucleotide of the primer is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence.

Other destabilising components may be incorporated along with the -2 or -3 nucleotide.

The diagnostic primer of the invention is preferably at least 20 nucleotides in length, most preferably 26 nucleotides in length but this may be between 15 and 20 nucleotides in length.In a further particularly preferred embodiment of the above aspect of the invention we provide diagnostic primers capable of binding to a template comprising wild type fungal cytochrome *b* nucleotide sequence wherein the final 3' nucleotide of the primer corresponds to a nucleotide present in a wild type fungal cytochrome *b* and wherein up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides may be varied with respect to the wild type sequence without significantly affecting the properties of the diagnostic primer.

In a further particularly preferred embodiment of this aspect of the invention we provide diagnostic primers comprising the sequences given below and derivatives thereof wherein the final nucleotide at the 3' end is identical to the sequences given below and wherein up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides may be varied without significantly affecting the properties of the diagnostic primer. Conveniently, the sequence of the diagnostic primer is exactly as provided below. In the majority of the primers listed below the penultimate nucleotide has been altered from wild type cytochrome *b* sequence to destabilise the primer thereby making it more selective for the desired template. It will be apparent to the man skilled in the art of primer design that bases alternative to or in addition to those discussed above may also be varied without adversely affecting the ability of the primer to bind to the template.

Primer	Species	primer sequence for the detection of WT sequence (5' to 3')
1	*Plasmopara viticola*	CCTTGGTGACAAATGAGTTTTTGG**A**G (SEQ ID NO 43)
2	*Erysiphe graminis f.sp. tritici/hordei*	CCATACGGGCAGATGAGCCACTGG**A**G (SEQ ID NO 44)
3	*Rhynchosporium secalis*	CCTTATGGACAGATGTCTTTATGA**T**G (SEQ ID NO 45)
4	*Pyrenophora teres*	CCCTACGGGCAAATGAGCCTTTGA**A**G (SEQ ID NO 46)
5	*Mycosphaerella graminicola*	CCTTATGGTCAAATGTCTTTATGA**A**G (SEQ ID NO 47)
6	*Mycosphaerella fijiensis* var.*difformis*	CCTTATGGTCAAATGTCTTTATGA**T**G (SEQ ID NO 48)
7	*Sphaerotheca fuliginea*	CCCTTCGGTCAAATGTCGCTCTGG**A**G (SEQ ID NO

		49)
8	*Uncinula necator*	CCCTACGGGCAGATGAGCCTATGGTG (SEQ ID NO 50)
9	*Colletotrichum graminicola*	CCTTACGGACAAATGTCATTATGAAG (SEQ ID NO 51)
10	*Pythium aphanidermatum*	CCTTGGTGTCAAATGAGTTTTTGGAG (SEQ ID NO 52)
11	*Colletotrichum gloeosporioides*	CCTTATGGACAAATGTCATTATGAAG (SEQ ID NO 53)
12	*Oidium lycopersicum*	CCCTACGGGCAGATGAGCCTGTGGAG (SEQ ID NO 54)
13	*Leveillula taurica*	CCATACGGACAAATGTCATTATGAAG (SEQ ID NO 55)
14	*Pseudoperonospora cubensis*	CCTTGGGGACAAATGAGTTTTTGGAG (SEQ ID NO 56)
15	*Alternaria solani*	CCTTATGGGCAAATGTCTTTATGAAG (SEQ ID NO 57)
16	*Cercospora arachidola*	CCTTATGGACAAATGTCATTATGAAG (SEQ ID NO 58)
17	*Rhizoctonia solani*	CCATACGGGCAAATGTCTCTGTGGAG (SEQ ID NO 59)
18	*Venturia inaequalis*	GTGTATGGTCAAATGAGCCTATGGAG (SEQ ID NO 60)
19	*Magnaporthe grisea*	CCTTATGGACAGATGTCATTATGAAG (SEQ ID NO 61)
20	*Phytophthora infestans*	CCTTGGGGACAAATGAGTTTTTGGAG (SEQ ID NO 62)
21	*Mycosphaerella musicola*	CCTTATGGTCAAATGTCTTTATGATG (SEQ ID NO 63)

Table 5: ARMS primer design for the detection of the wild type sequence

To adapt the primers shown in the above Table for use in a standard ASPCR reaction the last base at the 3' end should correspond to the wild type sequence without a destabilising base introduced.

The examples described above relate to ARMS primers based on the forward strand of DNA. The use of ARMS primers based on the forward strand of DNA is particularly preferred.

ARMS primers may also be based on the reverse strand of DNA if so desired. Such reverse strand primers are designed following the same principles above for forward strand primers namely, that the primers may be at least 20 nucleotides in length most preferably 26 nucleotides in length, but may be between 15 and 20 nucleotides in length and the final nucleotide at the 3' end of the primer matches the relevant template i.e. mutant or wild type and preferably the penultimate residue is optimally changed such that it does not match the relevant template. Additionally up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides in the primer may be varied without significantly affecting the properties of the diagnostic primer.

In many situations, it will be convenient to use a diagnostic primer of the invention with a further amplification primer referred to herein as the common primer, in one or more cycles of PCR amplification. A convenient example of this aspect is set out in European patent number EP-B1-0332435. The further amplification primer is either a forward or a reverse common primer. For each species, the primer used is as below. The Primers shown below are reverse primers.

	Species	primer sequence (5' to 3')
1	*Plasmopara viticola*	GATACCTAATGGATTATTTGAACCTACCT (SEQ ID NO 64)
2	*Erysiphe graminis f.sp. tritici/hordei*	AACACCTAAAGGATTACCAGATCCTGCAC (SEQ ID NO 65)
3	*Rhynchosporium secalis*	TACACCTAAAGGATTACCTGACCCTGCAC (SEQ ID NO 66)
4	*Pyrenophora teres*	TTCAAGTACATCCAATTTCAATATACACT (SEQ ID NO 67)
5	*Mycosphaerella graminicola*	TAACAGAAAATCCACCTCATACGAATTCAACTATG TCTTG (SEQ ID NO 68)
6	*Mycosphaerella fijiensis* var. *difformis*	AAACCTCCTCAAATAAACTCAACTATATC (SEQ ID NO 69)
7	*Sphaerotheca fuliginea*	TAACTGAGAAACCCCCTCAGAGAAACTCCACAATA TCTTG (SEQ ID NO 70)
8	*Uncinula necator*	TTACAGAAAAACCACCTCAAAGAAACTCCACGATA

		TCTTG (SEQ ID NO 71)
9	*Colletotrichum graminicola*	TAACTGAGAAACCTCCTCAAACGAATTCAACAATA TCTTG (SEQ ID NO 72)
10	*Pythium aphamdermatum*	CTACAGCAAATCCCCCCCATAACCAATCAACAATA TCTTT (SEQ ID NO 73)
11	*Colletotrichum gloeosporioides*	TAACAGAGAAACCTCCTCAAACGAATTCAACTATA TCTTG (SEQ ID NO 74)
12	*Oidium lycopersicum*	TTACAGAAAAACCTCCTCAAAGAAACTCCACGATA TCTTG (SEQ ID NO 75)
13	*Leveillula taurica*	TTACAGAGAAACCTCCTCAAATAAATTCAACTATA TCTTG (SEQ ID NO 76)
14	*Pseudoperonospora cubensis*	CTACAGCAAAACCGCCCCACAACCAATCAACAATA TCTTT (SEQ ID NO 77)
15	*Alternaria solani*	TAACACTGAAACCTCCTCAAATGAACTCAACAATA TCTTG (SEQ ID NO 78)
16	*Cercospora arachidola*	AAACAGAGAAACCTCCTCATATAAATTCAACTAAA TCTTG (SEQ ID NO 79)
17	*Rhizoctonia solani*	ACACGGAAAAGCCACCCCAGATTAACTCTACAAAA TCTTG (SEQ ID NO 80)
18	*Venturia inaequalis*	ATTGACTTAAGCCTCCCCACAGAAATTCGACTATA TCTTG (SEQ ID NO 81)
19	*Magnaporthe grisea*	TAACAGAAAAACCACCTCAAATGAATTCAACAATA TCTTG (SEQ ID NO 82)
20	*Phytophthora infestans*	CAACAGCAAAACCTCCCCATAACCAATCAACAATA TCTTT (SEQ ID NO 83)
21	*Mycosphaerella musicola*	TAACAGAAAACCCACCTCAAATAAATTCAACTATA TCTTG (SEQ ID NO 84)

Table 6: Examples of common primers to use with ARMS primers

In the case of the longer sequences provided in Table 6 the skilled man will be able to use this information to design appropriate primers.

It will be evident to the man skilled in the art that the common primer can be any convenient pathogen specific sequence which recognises the complementary strand of the cytochrome *b* gene (or other gene of interest) lying 3' of the mutation selective primer.

The PCR amplicon size is preferentially 50 to 400bp long but can be from 30 to 2500bp long, or potentially from 30 to 10,000bp long.

A convenient control primer may be used which is designed upstream from the $G_{143}A$ position. It will be evident to the man skilled in the art that the control primer may be any primer which is not specific for the amplification of the mutation or wild type sequences. When using these primers along with the corresponding reverse ('common') primer described above, amplification will occur regardless whether the $G_{143}A$ point mutation is present or not.

Primer	Species	Control primer sequence (5' to 3')
1	*Plasmopara viticola*	GCCTTGGGGACAAATGAGTTTTTG (SEQ ID NO 85)
2	*Erysiphe graminis f sp. tritici/hordei*	GCCATACGGGCAGATGAGCCACTG (SEQ ID NO 86)
3	*Rhynchosporium secalis*	TCCTTATGGACAGATGTCTTTATG (SEQ ID NO 87)
4	*Pyrenophora teres*	ACCCTACGGGCAAATGAGCCTTTGAG (SEQ ID NO 88)
5	*Mycosphaerella graminicola*	TACCTTATGGTCAAATGTCTTTATGA (SEQ ID NO 89)
6	*Mycosphaerella fijiensis* var. *difformis*	GTTTTACCTTATGGTCAAATGTCTTTATG (SEQ ID NO 90)
7	*Sphaerotheca fuliginea*	TTCCCTTCGGTCAAATGTCGCTCTGG (SEQ ID NO 91)
8	*Uncinula necator*	TACCCTACGGGCAGATGAGCCTATGG (SEQ ID NO 92)
9	*Colletotrichum graminicola*	TACCTTACGGACAAATGTCATTATGA (SEQ ID NO 93)
10	*Pythium aphanidermatum*	TACCTTGGGGTCAAATGAGTTTTTGG (SEQ ID NO 94)
11	*Colletotrichum gloeosporioides*	TACCTTATGGACAAATGTCATTATGA (SEQ ID NO 95)
12	*Oidium lycopersicum*	TACCCTACGGGCAGATGAGCCTGTGG (SEQ ID NO 96)
13	*Leveillula taurica*	TACCATACGGACAAATGTCATTATGA (SEQ ID NO 97)
14	*Pseudoperonospora cubensis*	TACCTTGGGGACAAATGAGTTTTTGG (SEQ ID NO 98)

15	*Alternaria solani*	TTCCTTATGGGCAAATGTCTTTATGA (SEQ ID NO 99)
16	*Cercospora arachidola*	TACCTTATGGACAAATGTCATTATGA (SEQ ID NO 100)
17	*Rhizoctonia solani*	TTCCATACGGGCAAATGTCTCTGTGG (SEQ ID NO 101)
18	*Venturia maequalis*	ACGTGTATGGTCAAATGAGCCTATGG (SEQ ID NO 102)
19	*Magnaporthe grisea*	TACCTTATGGACAGATGTCATTATGA (SEQ ID NO 103)
20	*Phytophthora infestans*	TACCTTGGGGACAAATGAGTTTTTGG (SEQ ID NO 104)
21	*Mycosphaerella musicola*	TACCTTATGGTCAAATGTCTTTATGA (SEQ ID NO 105)

Table 7: Examples of possible control primer design

A variety of methods may be used to detect the presence or absence of diagnostic primer extension products and/or amplification products. These will be apparent to the person skilled in the art of nucleic acid detection procedures. Preferred methods avoid the need for radiolabelled reagents. Particularly preferred detection methods are those based on fluorescence detection of the presence and/or absence of diagnostic primer extension products. Particular detection methods include gel electrophoresis analysis, "Scorpions"™ product detection as described in PCT application number PCT /GB98/03521 filed in the name of Zeneca Limited on 25 November 1998 the teachings of which are incorporated herein by reference, "Taqman" ™ product detection, for example as described in patent numbers US-A-5487972 & US-A-5210015; "Molecular Beacons" ® product detection, outlined in patent number WO-95/13399 and surface enhanced Raman resonance spectroscopy (SERRS), outlined in patent application WO 97/05280. Further preferred detection methods include ARMS linear extension (ALEX) and PCR with ALEX as described in published PCT application number WO 99/04037. Conveniently, real-time detection is employed. The use of "Scorpions"™ product detection as described in PCT application number PCT /GB98/03521 and published UK patent application No. GB2338301 is particularly preferred for use in all aspects of the invention described herein. The combination of the ARMS and the Scorpion technology as described herein is

particularly preferred for use in all aspects of the invention described herein and the preferred detection method is a fluorescence based detection method. Many of these detection methods are appropriate for quantitative work using all of the above primers. These different PCRs can be carried out in different tubes or multiplexed in one tube. Using such methods, estimates can be made on the frequency of point mutation molecules present in a background of wild type molecules.

As exemplified herein we have used ARMS primers based on the forward strand of DNA in combination with Scorpion detection based on the reverse strand of DNA as the detection method. The Scorpion detection element preferably comprise the reverse primers shown in Table 6. It will be readily apparent to the man skilled in the art that alternative combinations of ARMS primers and Scorpion detection elements could also be used. For example the primer based on the forward strand of the DNA could be a combination of an ARMS primer with a Scorpion detection system and this could be used with a common primer based on the reverse strand of DNA or the primer based on the reverse strand of DNA could be a combination of an ARMS primer with a Scorpion detection system and this could be used with a common primer based on the forward strand of DNA.

In most of the examples described herein, the Scorpion detection element is on the common primer. The ARMS primer specific to the mutation and the wild type sequence are used in combination with the common fluorescence labelled primer. These two reactions are carried out in different PCR tubes and the fluorescence is emitted when the probe binds to the amplicon generated. The Scorpion element may alternatively be incorporated on the ARMS primers. In this case, the two ARMS primers can be labelled with different fluorophores and used along with the common primer (this time unlabelled). These three primers may be included in the same reaction as the resulting mutant and wild type amplicons will lead to different fluorescence being emitted.

As described in published UK patent application No. GB2338301 the Scorpions technology may be used in a number of different ways such as the intercalation embodiment where the tail of the Scorpions primer carries an intercalating dye which is capable of being incorporated between the bases of a double stranded nucleic acid molecule, upon which it becomes highly fluorescent; the FRET embodiment where the dyes involved in the primer form an energy transfer pair; the no-quencher embodiment where a fluorophore is attached to the tail of the Scorpions primer; the Bimolecular embodiment where the fluorophore and

quencher may be introduced on two separate but complementary molecules: the Capture Probe embodiment where amplicons may be specifically captured and probed using the same non-amplifiable tail and the Stem embodiment where the primer tail comprises self complementary stems. These embodiments are described fully in published UK patent application No. GB2338301, the teaching of which is incorporated herein by reference.

The methods of the invention described herein reliably detect a mutation in a fungal gene wherein the presence of said mutation gives rise to fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene at a detection level in the range of 1 mutated allele per 1,000,000 wild type alleles to 1 mutated allele per 10,000 wild type alleles, and preferably in the range of 1 mutated allele per 100,000 wild type alleles to 1 mutated allele per 10,000 wild type alleles. The method of the invention can also detect mutations occurring at higher frequency, for example, 1 mutated allele per 100 wild type alleles, 1 mutated allele per 10 wild type alleles or where only mutated alleles are present. Similarly the methods of the invention may be used to detect the frequency of the wild type allele in a background of mutated alleles.

The combination of allele specific primer extension made more sensitive with use of the ARMS technology and quantitative detection methods which are used in the present invention make this an extremely valuable technique for the detection of fungal mutations occurring at low frequency.

The detection of alleles present in given isolates enables the results of phenotypic bioassays to be related to the DNA profile of the target gene. The discovery of a single point mutation as the resistance mechanism explains the qualitative nature of the resistance, and the confirmation of single spore isolate sequences validates the accuracy of the screens in determining frequencies of resistant and sensitive isolates in the samples tested.

The development of the method combining allele specific primer extension, the specificity of ARMS and real time fluorescent detection as exemplified herein with the Scorpion system enables a greater number of samples to be analysed for the presence of the resistance mutation than would be feasible in a bioassay programme. Larger sample numbers enable the identification of the resistance mutation at frequencies of a lower percentage than might be easily detected through bioassay. This enables resistance to be identified in the population before it might be apparent from field data. The high throughput nature of the method enables a wider area and more sites to be sampled and tested than might be possible

using the bioassay. Allele specific primer extension such as ARMS linked with real time fluorescent detection allows the detection of the presence of the resistance gene in a population before the effects of the gene can be viewed phenotypically by bioassay in heteroplasmic and/or heterokaryotic cells, thus reducing the error of classifying samples as sensitive when they carry a low frequency of the resistance genotype. Results are obtained much faster through simultaneous read-out (real time) compared to waiting for disease development *in planta*, enabling fast responses to field situations and advice on resistance management to be given more quickly.

One or more of the diagnostic primers of the invention may be conveniently packaged with instructions for use in the methods of the invention and appropriate packaging and sold as a kit. The kits will conveniently include one or more of the following: diagnostic, wild type, control and common oligonucleotide primers: appropriate nucleotide triphosphates, for example dATP, dCTP, dGTP, dTTP, a suitable polymerase as previously described, and a buffer solution.

In a further aspect the invention provides a method of detecting plant pathogenic fungal resistance to a fungicide said method comprising detecting a mutation in a fungal gene wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of said mutation in fungal nucleic acid using any (or a) single nucleotide polymorphism detection technique.

In a further embodiment of this aspect the invention provides a method of detecting plant pathogenic fungal resistance to a fungicide said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to presence or absence of a mutation in said nucleic acid wherein the presence of said mutation gives rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene.

In a preferred embodiment of this aspect the invention provides a method of detecting plant pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene comprising contacting a test sample comprising fungal nucleic acid with a diagnostic primer for a specific mutation, the presence of which gives rise to resistance to

said fungicide, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

In a further preferred embodiment of this aspect the invention provides a method of detecting plant pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene comprising contacting a test sample comprising fungal nucleic acid with a diagnostic primer for a specific mutation, the presence of which gives rise to resistance to said fungicide, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

The methods of the invention described in the above aspect and embodiments are especially suitable for use with plant pathogenic fungal strains where the presence of a mutation in a cytochrome *b* gene gives rise to fungicide resistance and most especially to resistance to a strobilurin analogue or a compound in the same cross resistance group and most especially where the mutation in the fungal DNA gives rise to a replacement of a glycine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143, more especially to a $G_{143}A$ replacement in the encoded protein, and especially where the mutation is a G to C base change at the second position in the codon at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143.

In a further aspect the invention provides a method of detecting and quantifying the frequency of a mutation giving rise to plant pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene said method comprising detecting the presence or absence of a mutation in a fungal gene wherein the presence of said mutation gives rise to fungal resistance to said fungicide said method comprising identifying and quantifying the presence or absence of said mutation in fungal nucleic acid using any (or a) single nucleotide polymorphism detection technique.

In a further preferred embodiment of this aspect the invention provides a method of detecting and quantifying the frequency of a mutation giving rise to plant pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene said method comprising detecting the presence or absence of a mutation in a fungal gene wherein

the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying and quantifying the presence or absence of said mutation in fungal nucleic acid using any (or a) single nucleotide polymorphism detection technique.

In a further embodiment of this aspect the invention provides a method of detecting and quantifying the frequency of a mutation giving rise to plant pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid with appropriate primers in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to both the presence and absence of a mutation in said nucleic acid wherein the presence of said mutation gives rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene, and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of an amplicon generated during the PCR reaction.

In a preferred embodiment of this aspect the invention provides a method of detecting and quantifying the frequency of a mutation giving rise to plant pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene comprising contacting a test sample comprising fungal nucleic acid with diagnostic primers to detect both the presence and absence of a specific mutation in said nucleic acid, the presence of which gives rise to resistance to said fungicide, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primers relating to the absence and the presence of the specific mutation are extended when the appropriate fungal template is present in the sample; and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of diagnostic primer extension products.

In a further preferred embodiment of this aspect the invention provides a method of detecting and quantifying the frequency of a mutation giving rise to plant pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene comprising contacting a test sample comprising fungal nucleic acid with diagnostic primers to detect both the presence and absence of a specific mutation in said nucleic acid, the presence of which gives rise to resistance to said fungicide, in the presence of appropriate nucleotide

triphosphates and an agent for polymerisation, such that the diagnostic primers relating to the absence and the presence of the specific mutation are extended only when the appropriate fungal template is present in the sample; and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of diagnostic primer extension products.

The methods of the invention described in the above aspect and embodiments are especially suitable for use with plant pathogenic fungal strains where the presence of a mutation in a cytochrome *b* gene gives rise to fungicide resistance and most especially to resistance to a strobilurin analogue or a compound in the same cross resistance group and most especially where the mutation in the fungal DNA gives rise to a replacement of a glycine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143, more especially to a $G_{143}A$ replacement in the encoded protein, and especially where the mutation is a G to C base change at the second position in the codon at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143.

In a yet further aspect the invention provides a method of selecting an active fungicide and optimal application levels thereof for application to a crop comprising analysing a sample of a fungus capable of infecting said crop and detecting and/or quantifying the presence and/or absence of a mutation in a gene from said fungus wherein the presence of said mutation may give rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene and then selecting an active fungicide and optimal application levels thereof.

In a particularly preferred embodiment of this aspect of the invention the detection method comprises any (or a) single nucleotide polymorphism detection technique and is more preferably comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the specific mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product and the quantification is achieved by contacting a test sample comprising fungal nucleic acid with diagnostic primers to detect both the presence and absence of a specific mutation in said nucleic acid the presence of which gives rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene in the presence of appropriate

nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primers relating to the absence and the presence of the specific mutation are extended when the appropriate fungal template is present in the sample; and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of diagnostic primer extension products.

In a further particularly preferred embodiment of this aspect of the invention the detection method comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the specific mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product and the quantification is achieved by contacting a test sample comprising fungal nucleic acid with diagnostic primers to detect both the presence and absence of a specific mutation in said nucleic acid the presence of which gives rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primers relating to the absence and the presence of the specific mutation are extended only when the appropriate fungal template is present in the sample; and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of diagnostic primer extension products.

The methods of the invention described herein are especially suitable for use with plant pathogenic fungal strains where the presence of a mutation in a cytochrome *b* gene gives rise to fungicide resistance and most especially to resistance to a strobilurin analogue or a compound in the same cross resistance group and most especially where the mutation in the fungal DNA gives rise to a replacement of a glycine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143, more especially to a $G_{143}A$ replacement in the encoded protein, and especially where the mutation is a G to C base change at the second position in the codon at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143

In a still further aspect the invention provides a method of controlling fungal infection of a crop comprising applying a fungicide to the crop wherein said fungicide is selected according to any of the selection methods of the invention described above.

The method of the invention described above is especially suitable for use with plant pathogenic fungal strains where the presence of a mutation in a cytochrome *b* gene gives rise to fungicide resistance and most especially to resistance to a strobilurin analogue or a compound in the same cross resistance group.

In a yet further aspect the invention provides an assay for the detection of fungicidally active compounds comprising screening the compounds against strains of fungi which have been tested for the presence or absence of a mutation giving rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene according to the methods of the invention described herein and then determining fungicidal activity against said strains of fungi.

The methods of the invention described herein are especially suitable for use with plant pathogenic fungal strains where the presence of a mutation in a cytochrome *b* gene gives rise to fungicide resistance and most especially to resistance to a strobilurin analogue or a compound in the same cross resistance group and most especially where the mutation in the fungal DNA gives rise to a replacement of a glycine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143, more especially to a $G_{143}A$ replacement in the encoded protein, and especially where the mutation is a G to C base change at the second position in the codon at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143.

By applying the methods of the invention described herein the appropriate rate of application of fungicides and/or the appropriate combination of fungicides to be applied to the crop may be determined.

The methods of the invention described herein are particularly suitable for monitoring fungal resistance to a strobilurin analogue or a compound in the same cross resistance group in crops such as cereals, fruit and vegetables such as canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion, vines and turf.

The methods of the invention described herein are particularly sensitive for detecting low frequencies of mutations in mitochondrially encoded genes, such as the cytochrome *b* gene, making this an especially useful and commercially important way of screening plant pathogenic fungi for the onset of fungicidal resistance wherein the resistance is due to a mutation in a mitochondrially encoded gene.

Brief Description of the Drawings

The invention will now be illustrated with reference to the following non-limiting Examples and Figures in which :

Figure 1 shows: a diagrammatic representation of the secondary structure of the Scorpion *P. viticola* primer using the MFold Zucker program

Figure 2a shows: a graph illustrating the PCR amplification of a serial dilution of *P.viticola* mutant DNA in a background of wild type DNA using the C specific primer and the Scorpion primer (in duplicate). As indicated on the figure the diamonds representing 50% C/G = line 1, the diamonds representing 100% G=line 7, the circles representing 10%C =line 2, the circles representing 1%C =line 3, the circles representing 0.1%C =line 4, the circles representing 0.01%C =line 5 and the circles representing NTC=line 6.

Figure 2b shows: a graph illustrating a multiplex experiment with *P. viticola* ARMS primers on two serial dilutions of mutant DNA in a background of wild type DNA (in duplicate). As indicated in the figure the line represented by the diamonds shows the results for 1:100 Gfam, the line represented by the triangles shows the results for 1:500 Gfam, the line represented by the circles shows the results for 1:100 Ctet, and the line represented by the squares shows the results for 1:500 Ctet.

Figure 3a shows: a graph illustrating *E. graminis* total DNA amplified with the three primer pairs (specific G/C and control primers) (in duplicate). As shown in the figure the line represented by the diamonds shows the results for 1/100 standard, the line represented by the triangles shows the results for 1/100 Gmix and the line represented by the crosses shows the results for 1/100 Cmix.

Figure 3b shows: a graph illustrating the amplification of a sensitive *E. graminis* isolate with the three primer pairs. As shown in the figure the line represented by the diamonds shows the results for Qmix,1/100 =line 1, the line represented by the squares shows the results for Qmix,1/1000 =line 2, the line represented by the triangles shows the results for Gmix,1/100 =line 3, the line represented by the circles shows the results for Gmix,1/1000 =line 4, the line represented by the cross shows the results for Cmix,1/100 =line 5, the line represented by the diamonds shows the results for Cmix,1/1000 =line 6.

Figure 4 shows: a graph illustrating the amplification of a resistant *E. graminis* isolate with the three primer pairs. As shown in the figure the line represented by the diamonds shows the results for Qmix,1/100 =line 1, the line represented by the squares shows the results for

Qmix,1/1000 =line 2, the line represented by the triangles shows the results for Gmix,1/100 =line 3, the line represented by the diamonds shows the results for Gmix,1/1000 =line 4, the line represented by the cross shows the results for Cmix,1/100 =line 5, the line represented by the circles shows the results for Cmix,1/1000 =line 6.

Figure 5a shows: a graph illustrating the PCR amplification of a serial dilution of a wild type *R. secalis* plasmid amplified with the wild type specific primer pair (in duplicate). As shown in the figure the line represented by the diamonds shows the results for Gplasmid 10^8, the line represented by the triangles shows the results for G-plasmid 10^6, the line represented by the squares shows the results for G-plasmid 10^4 and the line represented by the circles shows the results for G-plasmid 10^2.

Figure 5b shows: a graph illustrating the PCR amplification of the highest concentration of wild type and mutant *R. secalis* plasmids amplified with the wild type specific primer pair (in duplicate). As can be seen from the figure the line represented by the diamonds shows the results for G-plasmid 10^8 and the line represented by the triangles shows the results for c-plasmid 10^8.

Figure 6a shows: a graph illustrating the PCR amplification of a serial dilution of a mutant *R. secalis* plasmid amplified with the mutant specific primer pair (in duplicate). As shown in the figure the line represented by the diamonds shows the results for c-plasmid 10^8, the line represented by the triangles shows the results for c-plasmid 10^6, the line represented by the squares shows the results for c-plasmid 10^4 and the line represented by the circles shows the results for c-plasmid 10^2.

Figure 6b shows: a graph illustrating the PCR amplification of the highest concentration of wild type and mutant *R. secalis* plasmids amplified with the mutant specific primer pair (in duplicate). As can be seen from the figure the line represented by the diamonds shows the results for G-plasmid 10^8 and the line represented by the triangles shows the results for c-plasmid 10^8.

Figures 7a, b and c show: graphs illustrating the PCR amplification of *R. secalis* DNA and cDNA templates in three dilutions with the G primer pair (in duplicate). As can be see from the figures the line represented by the diamonds shows total DNA and the line represented by the triangles shows cDNA.

Figures 8 a, b and c show: graphs illustrating the PCR amplification of *R. secalis* DNA and cDNA templates in three dilutions with the C primer pair (in duplicate). As can be see from

the figures the line represented by the diamonds shows total DNA and the line represented by the triangles shows cDNA.

Figure 9a and b show: graphs illustrating the amplification of *P. teres* P13 and P15 isolates in two dilutions with the three primer pairs (in duplicate). As indicated on the figure in Figure 9a the diamonds representing S-mix = line 1, the triangles representing S-mix 1/10 =line 2, the squares representing G-mix =line 3, the circles representing G-mix 1/10 =line 4, the circles representing C-mix =line 5 and the squares representing C-mix 1/10 =line 6. In figure 9b the diamonds representing s-mix = line 1, the triangles representing s-mix,1/10=line 2, the squares representing g-mix =line 3, the circles representing g-mix,1/10 =line 4, the circles representing c-mix =line 5 and the squares representing c-mix,1/10 =line 6.

Detailed Description of the Invention

EXAMPLES:

In the first four Examples below the Scorpion™ system (AstraZeneca Diagnostics) was used as a product detection system. This detection system is described in full in PCT application number PCT/GB98/03521 filed in the name of Zeneca Limited on 25 November 1998 the teachings of which are incorporated herein by reference. This novel detection system uses a tailed primer and an integrated signalling system. The primer has a template binding region and a tail comprising a linker and a target binding region. In use the target binding region in the tail hybridises to complementary sequence in an extension product of the primer. This target specific hybridisation event is coupled to a signalling system wherein hybridisation leads to a detectable change. The detection method of this system offers a number of significant advantages over other systems. Only a single primer/detector species is required. This provides both increased simplicity and enhanced specificity based on the ready availability of the target binding region for hybridisation with the primer extension product. The newly synthesised primer extension product is the target species so the output signal obtainable is directly related to amount of extended primer. It is not dependent on additional hybridisation events or enzymatic steps. Intra and inter-strand competition for the probe site is limited so probe design becomes simplified. As the interaction is unimolecular, the signalling reaction is very rapid, permitting increased cycling rates which is a significant feature for experimental efficiency.

The Scorpion primers designed in the examples described below have the following modifications in common:

- A hexethylene glycol (HEG) monomer as a blocking moiety that is sited between the template binding region of the primer and the tail region, which moiety prevents polymerase mediated chain copying of the tail region of the primer template.
- A FAM fluorescent molecule is added to the 5'end of the primer. FAM is one of the fluorescence molecules that can, for example, be readily detected by the 488nm laser of the ABI PRISM 7700 instrument (PE Biosystems)
- MR is a non-fluorogenic quencher attached to a uracil residue

Other fluorescence molecules and quenching mechanisms can also be accommodated in Scorpion primer design and would be suitable to use in this invention.

In Example 4, an intercalating dye was also used as a detection mechanism. The eighteen examples together describe the characterisation of partial cytochrome *b* gene sequences from a panel of important wild type fungal isolates, whilst Examples 1, 2 and 6 also describe the characterisation of partial cytochrome *b* gene sequences from fungal isolates which are resistant to strobilurin analogues and other compounds in the same cross resistance group. The methods are particularly suitable for use with the strobilurin analogues azoxystrobin and picoxystrobin.

EXAMPLE 1

In Example 1, we describe the characterisation of partial *Plasmopara viticola* cytochrome *b* gene sequence, the characterisation of a single nucleotide polymorphism (SNP) that gives rise to strobilurin analogue resistance in *P. viticola* and the validation of a real time PCR Scorpion assay for the monitoring of this SNP in *P. viticola*. A multiplexing approach to carrying out the real time PCR assay is also described. *P. viticola* is the causal agent of vine downy mildew.

The wild type ES2B strobilurin analogue sensitive isolate of *P. viticola* was collected in 1996 from Spain. This isolate had never been exposed to strobilurin analogue selection. Infected vine leaves were hand picked and stored in a polythene bag and sent to Jealott's Hill Research Station (Zeneca Agrochemicals). Upon arrival the leaves were placed in pairs, with abaxial sporulating surfaces together, over moist absorbent paper in plastic boxes, and incubated for 24-48 hours at 21-24°C. Single lesions were excised from the leaves and the

sporangia suspended in 5ml deionised water, then sprayed onto the abaxial surfaces of 5-6 week old vine seedlings (var. Ohanez). Freshly inoculated plants were incubated for 24 hours in a humidity chamber (temperature ambient, relative humidity 100%), then moved to a growth room (day 24°C/r.h. 60%; night 18°C/r.h. 95%; daylength 16 hours; 6,000 lux). Plants were returned to the humidity chamber after 6 days for a further 24 hour period, after which time successful infection showed as sporulating lesions on the abaxial leaf surfaces. Further subculturing was carried out as above, adjusting the sporangial suspension to 5,000-10,000 sporangia per ml.

A DNA fragment encoding a significant part of wild type *P. viticola* cytochrome *b* sequence was amplified using primers based on conserved regions of *Phytophthora megasperma* and *Aspergillus nidulans* cytochrome *b* genes (Cytb12F (5' TGAACATATTATGAGAGATGT 3') (SEQ ID NO 106)and Cyt10R (5' AATTGCATAAAAAGGTAAAAA 3') (SEQ ID NO 107) which delineate the sequence encoding amino acid region 66 and 281 of fungal cytochrome *b* based on the *S.cerevisiae* numbering system). DNA was extracted from the strobilurin analogue-sensitive isolate, using a phenol/chloroform extraction protocol. Sporangia were washed into 30ml of double distilled H_2O (ddH_2O) from six leaves with 90-100% disease cover (originated from artificially inoculated six week old vine seedlings). The sporangial suspension was filtered through Miracloth (Calbiochem cat # 475855) and centrifuged at 3600rpm for 10 minutes at 4°C. The sporangia were then frozen in liquid nitrogen and ground to a fine powder using a pestle and mortar which had been previously cleaned and sterilised by acid washing and autoclaving. 0.5ml of lysis buffer (200mM Tris-HCl (pH8.5), 250mM NaCl, 25mM EDTA and 0.5% SDS) was added and the suspension was transferred to a sterile screw cap 1.5ml Eppendorf tube. 0.5ml of a phenol/chloroform/isoamyl alcohol (25:24:1) mixture was immediately added and mixed by inverting several times. The tubes were centrifuged for 30 minutes at 14000rpm and the aqueous phase transferred to fresh Eppendorf tubes. The phenol/chloroform/isoamyl alcohol extraction was then repeated but this time the tubes were centrifuged at 14000rpm for only 15 minutes. After transferring the aqueous phase to a new Eppendorf tube, a final chloroform extraction was performed. Fungal DNA was then precipitated by adding 0.1 vol of 3M sodium acetate (pH5.5) and 0.6 vol of isopropanol. After inverting several times, the tubes were centrifuged at 14000rpm for 20 minutes at 4°C. The DNA pellet was then washed twice with 70% ethanol, vacuum dried and resuspended in

50µl of ddH_2O. DNA yield and quality was checked by gel electrophoresis and a serial dilution (1:10, 1:100 and 1:1000) was made in ddH_2O for use as template material in subsequent PCRs. PCRs were set up as recommended by the manufacturer of the Taq Polymerase enzyme (Gibco) in a final reaction volume of 100µl. The primers were added to the reactions to a final concentration of 1pmole/µl. 10µl of each DNA dilution was added to the appropriate PCRs. Standard procedures were carried out in order to limit the risk of PCR contamination. 30 cycles of 94°C for 45 sec, 42°C for 45 sec and 72°C for 1 min 30 sec were carried out on a Hybaid Omn-E PCR instrument. An initial step at 94°C for 3 minutes and a final extension at 72°C for 10 minutes were also performed. The efficiency of the PCRs was then assessed by analysing 18µl of the reaction mixtures by gel electrophoresis. A 2µl sample of the ~500bp PCR products was then cloned in the TA PCR cloning pCR2.1 vector (Invitrogen Catalogue No. K4500-01) and transformed into *Escherichia coli* cells (TOP10 One Shot™ competent cells) as per the manufacturer's recommendations. A series of resulting clones were checked for the presence of inserts by performing Wizard minipreps (Promega catalogue No. A7100) as per manufacturer's instructions and restriction digest analysis using *Eco*RI. 6 clones with suitable inserts (~500bp) were then sequenced using M13 forward and reverse primers (ABI377XL automated sequencer). When the nucleotide sequence data from these studies was analysed using the relevant bioinformatics software (Seqman, Editseq and Macaw), the resulting novel sequence was found to encode a new cytochrome *b* gene with close homology to other known oomycete cytochrome *b* sequences such as *P. megasperma*. The sequence of a 61 nucleotide tract of the resultant *P. viticola* encoding 30bp upstream and downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in Table 3.

P. viticola specific primers based on the above analyses used in later amplifications of the cytochrome *b* region of interest were PLAS17F (5' AAATAACGGTTGGTTAATTCG 3') (SEQ ID NO 108) and PLAS15R (5' TCTTAAAATTGCATAAAAAGG (SEQ ID NO 109)3') delineating amino acid region 73-283 of fungal cytochrome *b* according to the *S.cerevisiae* numbering system.

A strobilurin analogue-resistant isolate of *P. viticola* was identified at one trial site. Infected vine leaves were collected and processed essentially as above though samples were subcultured as mass populations and not isolated as single lesions prior to testing. The test method to verify strobilurin analogue resistance was a 24 hour preventative spray on 4-week

old vine seedlings. A chemical dilution series was prepared by dissolving 5mg strobilurin analogue (strobilurin analogue as used in all examples herein denotes azoxystrobin) (technical material, 97% pure) in 1ml acetone and carrying out a further dilution in deionised water at room temperature to give a rate of 10ppm (a dose known to give 100% control of strobilurin analogue sensitive baseline isolate). The abaxial surfaces of the target leaves were sprayed using a DeVilbiss spray gun, 10psi, to maximum retention. Control plants were sprayed with deionised water only. The treated plants were left to dry in a growth room (conditions as above) overnight. Inoculation of the test *P. viticola* sample was carried out at 5,000 sporangia per ml and freshly inoculated plants were incubated as described previously. 7 days following inoculation any potentially resistant growth on the treated leaves was isolated and subcultured to provide sufficient material for PCR analysis. This isolate was designated T5.

Partial cytochrome *b* gene sequence was amplified with PLAS17F and PLAS15R primers from the resistant isolate (T5). Total genomic DNA (nuclear and mitochondrial) from this isolate was extracted using the phenol/chloroform extraction protocol described above. Again DNA presence and quality was checked by gel electrophoresis and suitable aliquots were diluted 1:10 and 1:100 in ddH_2O for PCR studies. 10µl of each DNA dilution was then added to Ready.To.Go™ Taq polymerase PCR beads (Amersham Pharmacia Biotech product number 27-9555-01) and made up to 25µl with PLAS17F and PLAS15R primer solutions, each to a final primer concentration of 1pmole/µl. Standard procedures were carried out to limit the risk of PCR contamination. 30 cycles PCR was then carried out at 94°C for 45sec, 52°C for 45 sec and 72°C for 1 min 30 sec on a Hybaid Omn-E PCR instrument. An initial step at 94°C for 3 minutes and a final extension step at 72°C for 10 minutes were also included. These PCRs were performed in duplicate. After the analysis of 10µl of the PCRs by gel electrophoresis on an 0.8% TBE agarose gel, the resulting ~500bp PCR products were pooled prior to cloning. A 1µl sample of the pooled PCR products was then cloned in the TA cloning pCR2.1 vector (Invitrogen) and transformed into *E.coli* cells (TOP10 One Shot™ competent cells) as per the manufacturer's recommendations. A series of resulting clones were checked for the presence of inserts by performing Wizard minipreps (as per Promega instructions) and restriction digest analysis using *EcoRI*. 10 clones with the correct size inserts (~500bp) were then sequenced using M13 forward and reverse primers (ABI377XL automated sequencer).

Analysis of the sequence data using suitable bioinformatics software (Seqman, Editseq and Macaw software) in all 10 cases revealed a G-->C point mutation in the cytochrome *b* sequence when compared to wild type sequence. This DNA point mutation leads to a single glycine to alanine change at position 143 (according to the *S.cerevisiae* amino acid coding system).

Different specific ARMS *P.viticola* primers were designed to detect the presence or absence of this $G_{143}A$ point mutation:

two forward ARMS primers based on the wild type sequence:

G-sp-f-1: CCTTGGTGACAAATGAGTTTTTGTGG (SEQ ID NO 110)

G-sp-f-2: CCTTGGTGACAAATGAGTTTTTGGAG (SEQ ID NO 111)

two forward ARMS primers based on the $G_{143}A$ mutation:

C-sp-f-1: CCTTGGTGACAAATGAGTTTTTGGCC (SEQ ID NO 112)

C-sp-f-2: CCTTGGTGACAAATGAGTTTTTGGAC (SEQ ID NO 113)

and a control primer designed upstream from the point mutation:

STAND: GCCTTGGGGACAAATGAGTTTTTG (SEQ ID NO 114)

In all of the above ARMS primers, the -1 base (the 3'end base of the primer sequence) corresponds to the target point mutation site. Bases presented in bold differ from the wild type *P. viticola* cytochrome *b* sequence. In all of the ARMS primers (not the control primer), the -20 base was changed from a G to a T base. This was done to disrupt the run of G bases. In the G-sp-f-2 and C-sp-f-2 primers, the -2 position was changed from a G to a A base. In the G-sp-f-1, the -3 position was changed from a G to a T base. In the C-sp-f-1 primer, the -2 primer was changed from a G to a C base. These alterations to the sequence were made to destabilise the primer and render any primer extension more specific to the corresponding template. Examples in the literature have shown that destabilising the ARMS primer decreases the risk of the primer mispriming on the wrong template (Newton et al, Nucleic Acid Research 17 (7) 2503-2516 1989).

The Scorpion™ product detection system was used in this case as a detection mechanism and the detection system was incorporated on the reverse primer. The resulting amplicon was 234 bp long with the ARMS primers, and 235 bp long with the control primer. The Scorpion primer was designed using Oligo 5 and MFold programs (MFold predicts optimal and suboptimal secondary structures for RNA or DNA molecules using the energy minimization method of Zucker (Zucker, M. (1989) Science 244, 48-52; SantaLucia, J.Jr.

(1998). Proc. Natl. Acad. Sci. USA 95, 1460-1465). The sequence of the *P. viticola* Scorpion primer was:

5' FAM-CCCGCC**GTAATTGTAGGGGCTGTACTAATAC**GGCGGG (SEQ ID NO 115)

MR-HEG-*GATACCTAATGGATTATTTGAACCTACCT* 3'(SEQ ID NO 116)

Underlined regions are the hairpin forming parts, FAM is the fluorescein dye, MR is a non-fluorogenic quencher attached to a uracil residue and HEG is the replication blocking hexethylene glycol monomer. The sequence in italics is the reverse primer sequence and the sequence in bold is the Scorpion sequence that binds to the extension product of the reverse primer.

The stem loop secondary structure of this Scorpion primer can be visualised using the MFold program (see figure 1). It is predicted to have an energy of -2.2 kcal/mol in its inert form. However in the presence of the extension product the hairpin structure is separated, as the probe sequence of the Scorpion primer binds to the extension product with a predicted energy of -6.1 kcal/mol. This separates the FAM dye from its quencher, causing emission of fluorescence detectable, for example, by an ABI Prism 7700 instrument. The annealing of the Scorpion element onto the newly synthesised strand is therefore energetically favourable compared to the Scorpion stem loop configuration in its inert state.

All primers were synthesised by Oswel DNA Service (Lab 5005, Medical and Biological Sciences Building, Southampton). Before use, the primers were diluted to 5µM in a total volume of 500µl ddH_2O each. The primers were then further diluted to a final concentration of 500nM in the PCRs.

In all cases AmpliTaq Gold enzyme (Perkin-Elmer/ABI) was included in the reaction mix at 1unit/25µl reaction. The reaction mix also contained 1x buffer (10mM Tris-HCl (pH8.3), 50mM KCl, 3.5 mM $MgCl_2$, 0.01% gelatine) and 100µM dNTPs. Amplifications were performed in an ABI Prism 7700 instrument for continuous fluorescence monitoring. A preliminary cycle of 95°C for 10 minutes was performed followed by 50 cycles of 95°C for 15 sec and 60°C for 45 sec. Fluorescence was monitored during the annealing/extension stage throughout all cycles.

Primers were first validated for use in such analyses by using plasmid DNA as template at various concentrations. This was performed in order to check the specificity and sensitivity of the primer designs. Partial wild type cytochrome *b* gene sequence and the corresponding tract containing the $G_{143}A$ mutation were cloned into the TA pCR2.1 vector

(Invitrogen) for use in this validation process. The C-sp-f-2 and G-sp-f-2 primers were preferred to the C-sp-f-1 and the G-sp-1 primers as duplicate PCRs gave more consistent results and were slightly more specific. In all cases, plasmid DNA was diluted in 1mg/ml bovine serum albumin (BSA).

The graph shown in Figure 2a illustrates PCRs where a dilution of mutant plasmid in a background of wild type template was amplified using the ARMS C-sp-f-2 primer. As the C plasmid dilution reduces in the wild type plasmid background, the fluorescence detection is delayed. In all cases, the final plasmid concentration in the PCR was $1x10^7$ molecules/µl. With each 10 fold dilution of the C plasmid, there is a delay of 3-4 cycles in the detection of fluorescence. When the C plasmid is present at only 1 in 10000 copies in wild type plasmid background, it is still detected by the specific ARMS primer. The C-sp-f-2 primer is totally specific to its corresponding template as no fluorescence can be detected in the 100% wild type plasmid sample in this experiment.

The different possible materials that could be used as starting material for resistance monitoring assays using such Scorpion technology were investigated. Three different isolates were used in this study. For each of these isolates, sporangia were collected both directly from diseased field vine leaves and from artificially innoculated glasshouse-grown leaves (as described previously). In both cases the sporangia were washed off the leaves using ddH_20. The sporangia were then collected by centrifugation and maintained at -80°C until needed. The sporangial samples were then resuspended in 1ml of ddH_2O and diluted 1:10 and 1:100 again in ddH_20. Real time PCR assays were carried out as described above except that bovine serum albumin (0.25µg/µl) was also added to each PCR, using 5µl of each dilution as template. Each dilution was carried out in duplicate, with C-sp-f-2, G-sp-f-2 and the control forward primer each added together with the common Scorpion reverse primer. The use of sporangia collected from glasshouse-grown vine leaves as starting material gave good results with consistent cycle threshold values (Ct). Using sporangia collected directly from field vine leaves gave poor results so the following two approaches were followed in order to improve the quality of the data: to reduce the inhibition in the PCR (field material will contain many more possible contaminants compared to glasshouse-grown leaves) and to make the DNA more available for amplification.

To reduce the effect of inhibitory components on the PCR, the concentration of BSA added to the reactions was increased and the detergent Tween-20 was added to the PCR. To

make the DNA more available for amplification, an initial PCR was carried out and the PCR product from this reaction was used as a template in the real time PCR, the spore dilutions were boiled for 10 minutes before being added to the real time PCR to improve cell lysis and the DNA was extracted from the spores using 3 different protocols (DNA Isolator from Genosys Biotechnologies Inc., DNAzol from Helena Biosciences, and Qiagen DNeasy plant mini kit). Each method was tried in turn with sporangia or DNA diluted 1:10, 1:100 and 1:1000 in ddH_2O as template. Each dilution was tested in triplicate with the C-sp-f-2 and G-sp-f-2 ARMS primers and the control primer; all with the reverse Scorpion common primer. 5µl of each template was added to the PCR assays and the conditions were essentially as described previously but with addition of 0.25µg/µl BSA (when the BSA concentration was not a variable). DNA extracted using either DNAzol or Qiagen DNA preps gave good and consistent results.

The G_{143} and A_{143} allele frequency was estimated in a *P. viticola* isolate collected from a field trial (denoted 17A). Sporangia were collected in ddH_20 off 120 leaves and harvested by centrifugation. This sample contained approximately 2400 lesions and $6.4x10^7$ sporangia. $12x10^6$ sporangia were kept at -80°C to be used for DNA extraction. A genomic DNA extraction using the DNeasy Plant Minikit (Qiagen catalogue No. 69103) was carried out and the resulting DNA was diluted 1:10 and 1:100 in ddH_20 for use as template for real time PCR analysis. The PCR conditions were as described above. The resulting Cycle Threshold (Ct) values were 20 for the G specific primer and 34 with the C specific primer giving a Ct difference of 14 cycles and thus a frequency of resistant alleles of approximately 1 in 10,000.

A multiplex approach has also been evaluated. In this instance, the Scorpion detection element was incorporated on the ARMS primers to enable the G_{143} and A_{143} allele PCR amplifications to be multiplexed in the same PCR. The Scorpion/ARMS primer sequences were as follows:

G_{143} allele specific primer:

5'FAM CCCGCCC **TGGGATAGCCGAGAATAAAT** GGGCGGG (SEQ ID NO 1) MR-HEG *CCTTGGTGACAAATGAGTTTTTGGAG* (3' SEQ ID NO 118)

A_{143} allele specific primer:

5'TET CCCGCCC **TGGGATAGCCGAGAATAAAT** GGGCGGG (SEQ ID NO 119) MR-HEG *CCTTGGTGACAAATGAGTTTTTGAGC* 3'(SEQ ID NO 120)

The Scorpion detection element details are as described above. The A_{143} allele specific primer was labelled with TET to allow the distinction to be made between the two amplicons. A common reverse (unlabelled) primer was used:

Reverse 5' CATAACCAGTCAACAACTTCTTTTCC 3'(SEQ ID NO 121)

The amplicon generated in both cases was 95bp long. Again Scorpion primers were designed using the Oligo5 and MFold programs (as described above). The other real time PCR components were as described above with only the primer concentrations being varied during the validation of this multiplexing approach. By decreasing the final concentration of the G FAM Scorpion to 100nM and maintaining the final concentration of the C TET Scorpion and the reverse primer at 500nM, it was possible to reliably detect ratios of at least 1:500 C:G using plasmid DNA as template (see figure 2b).

EXAMPLE 2

In Example 2, we report the characterisation of partial *Erysiphe graminis* f.sp *tritici* and *hordei* cytochrome *b* gene sequences, the characterisation of a SNP that gives rise to resistance to strobilurin analogues or compounds in the same cross resistance group and the description of a real time PCR Scorpion assay for the monitoring of this SNP in *E. graminis* f.sp. *tritici* and *hordei*.

Isolates of *E. graminis* f.sp. *tritici* and f.sp. *hordei* (causal agents of wheat and barley powdery mildew) were collected from Northern France, Germany, Ireland and the United Kingdom. This was achieved by one of two methods: hand collection of field leaves or air spora sampling by car-mounted jet spore trap (Burkard Manufacturing Co. Ltd., Rickmansworth, UK).

Wheat leaves infected with sporulating powdery mildew were collected from sites where the populations had been exposed to strobilurin analogues in multiple trials. Upon return to the laboratory, the leaves were placed in polythene bags and incubated at 21°C overnight. The following day pustules were resporulating. All pustules were subcultured by tapping infected leaves above fresh leaf pieces (wheat cv Rapier, 9 days old) placed over filter paper (Whatman No. 1) in 9cm petri dishes containing 1.2% water agar. The freshly inoculated plates were incubated for 7 days and the resulting colonies were then tested for sensitivity to strobilurin analogues.

For spore trapping, wheat leaves were cut from 9 day old plants (cv. Rapier) and placed on 1.8% water agar in plastic dishes, and maintained at 5°C until required.

A jet spore trap was mounted on top of a car and the car was driven at speeds up to 90 km/hr along prearranged routes in each country. The plastic dishes containing the leaf pieces were placed in the base of the spore trap column to allow airborne spores entering the trap to settle onto the leaves. Dishes were changed approximately every 80 km. Once a batch of leaf pieces had been used in the spore trap, they were transferred to square petri dishes ($10cm^2$) containing 60ml 1.8% water agar and filter paper and stored at 5°C.

On return to Jealott's Hill, the leaves exposed in the spore trap were incubated in a constant temperature room (day length 16hrs, light 4-5,000 lux, temperature 21°C, relative humidity ambient). 5-6 days after exposure in the spore trap, *E. graminis* pustules could be seen forming (small areas of yellowing of the leaf material followed by appearance of powdery sporulating lesions). These were either subcultured onto leaf pieces in 9cm Petri dishes as "populations" (one population per sampling stage) or excised as single pustule isolates and incubated separately on leaf pieces in 5cm petri dishes on 15ml 1.2% water agar covered with filter paper. Leaf pieces inoculated with populations of *E. graminis* were incubated for 7 days after which time sporulation was sufficient to inoculate a phenotypic resistance assay. Single spore isolates were incubated for 7 days but subcultured one further time to provide enough material for testing. If sporulation was poor the above process was repeated until good (60-70%) sporulating disease coverage was obtained on all leaf pieces in order to generate sufficient conidia for assay.

Testing and subsequent maintenance of resistant isolates was carried out on detached wheat seedling leaves treated 24 hours prior to inoculation with an aqueous solution of strobilurin analogue at 5ppm (a rate known to give 100% control of strobilurin sensitive baseline isolates) and Tween 20 wetting agent. Isolates were tested either as mass populations or single pustule isolates. Conidia were dry-inoculated onto treated leaf pieces. Infected material was incubated in a controlled environment (as described above) for 7 days prior to assessment.

Any growth on leaf pieces treated with 5ppm strobilurin analogue was considered potentially resistant. Material from these lesions was further subcultured onto strobilurin analogue-treated leaves to confirm resistance *in planta*, and analysed using the molecular assay described in this application. Phenotypic resistance frequencies of approximately 1 in 100 and higher could be detected by the mass population screen, and more precise frequencies were estimated by comparing single spore isolates where growth on treated

leaves was comparable to controls (resistant), with plates where the treated leaves gave 100% control of disease (sensitive).

Partial *E. graminis* f.sp. *tritici* cytochrome *b* gene sequence was amplified using primers based on conserved regions of *Aspergillus niger* and *Neurospora crassa* cytochrome *b* genes (Cytb3F (5' CAGCTTCAGCTTTCTTCT 3') (SEQ ID NO 122) and Cytb9R (5' ACTTAAAGGTCTAAATTG 3') (SEQ ID NO 122) which delineate the sequence encoding amino acid region 86 to 322 of fungal cytochrome *b* based on the *S. cerevisiae* numbering system). Approximately 500mg conidia from a strobilurin analogue-sensitive isolate that had had no exposure to strobilurin analogue selection were collected by tapping directly off leaves with sporulating disease into 1.5ml Eppendorf tubes. DNA was extracted from this conidial sample using a phenol/chloroform extraction protocol (see above). DNA presence and quality was analysed by gel electrophoresis and a serial dilution (1:10, 1:100 and 1:1000) was made in ddH_2O for use as template material in PCRs. PCRs were set up as recommended by the manufacturer of the Taq Polymerase enzyme (Gibco) in a final volume of 100µl and primers were added to the reactions to a final concentration of 1pmole/µl. 10µl of each DNA dilution was added to the appropriate PCRs. Rigorous procedures were undertaken in order to limit the risk of PCR contamination. 30 cycles of 94°C for 45 sec, 42°C for 45 sec and 72°C for 1 min 30 sec were carried out in a Hybaid Omn-E instrument. An initial incubation at 94°C for 3 minutes and a final extension at 72°C for 10 minutes were also performed. The efficiency of the PCRs was then assessed by analysing 18µl of the reaction mixtures by gel electrophoresis. A 2µl sample of the ~500bp PCR products was cloned in the TA PCR cloning pCR2.1 vector (Invitrogen) and transformed into *E.coli* cells (TOP10 One Shot™ competent cells) as per the manufacturer's recommendations. A series of resulting clones was checked for the presence of inserts by performing Wizard minipreps (as per Promega instructions) and restriction digest analysis using *EcoRI*. 6 clones with expected inserts size (~500bp) were then sequenced using M13 forward and reverse primers (ABI377XL automated sequencer). When the nucleotide sequence data from these studies was analysed using the relevant bioinformatics software, the resulting novel sequence was found to encode a new cytochrome *b* gene with close homology to other known ascomycete cytochrome *b* sequences. A 61 nucleotide tract, encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system), can be found in Table 3.

E. graminis specific primers designed on the basis of the novel sequence were used in later amplifications of the cytochrome *b* region of interest. These were ERY11F (5' ATGAACAATTGGTACAGTAAT 3') (SEQ ID NO 124) and ERY12R (5' GTTAGGTATAGATCTTAATAT 3') (SEQ ID NO 125). Together they delineate the sequence encoding amino acids region 114-287 of fungal cytochrome *b* according to the *S. cerevisiae* coding system.

Partial cytochrome *b* sequence was amplified with ERY11F and ERY12R primers from an *E. graminis* f.sp. *tritici* strobilurin analogue-resistant population. Conidia (~200mg) were suspended in 200µl of ddH_2O and diluted 1:10, 1:100 and 1:1000 in ddH_2O. 10µl of each conidial dilution was added to a Ready.To.Go™ Taq polymerase PCR bead (Amersham Pharmacia Biotech product number 27-9555-01) and made up to 25µl with ERY11F and ERY12R primer solutions so that the final primer concentration was 1pmole/µl. Standard procedures were carried out to limit the risk of PCR contamination. 30 cycles of 94°C for 45 sec, 52°C for 45 sec and 72°C for 1 min 30 sec were carried out on a Hybaid Omn-E instrument. An initial step at 94°C for 3 minutes and a final extension at 72°C for 10 minutes were also included. All PCRs in this case were performed in triplicate. After the analysis of 10µl of the PCRs by gel electrophoresis on an 0.8% TBE agarose gel, the resulting ~500bp PCR products were pooled prior to cloning. A 2µl sample of the pooled PCR products was cloned in the TA Invitrogen PCR cloning pCR2.1 vector and transformed into *E.coli* (TOP10 One Shot™ competent cells) as per the manufacturer's recommendations. A series of clones was checked for inserts by performing Wizard minipreps (as per Promega instructions) and restriction digest analysis using *EcoRI*. 10 clones with inserts of the expected size (~500bp) were then sequenced using M13 forward and reverse primers (ABI377XL automated sequencer). Analysis of the sequence data generated using suitable bioinformatics software revealed a G-->C point mutation in the cytochrome *b* gene sequence in all 10 isolates when compared with the previously obtained wild type *E. graminis* f.sp. *tritici* cytochrome *b* gene sequence. This DNA point mutation leads to a glycine to alanine change at position 143 (according to the *S.cerevisiae* coding system).

Partial cytochrome *b* gene sequence was also characterised from two *E. graminis* f.sp. *hordei* isolates. Small samples of conidia (~100mg) were tapped off infected barley leaves (which were prepared as previously described for the wheat) into sterile Eppendorf tubes.

These conidial samples were kept at -80°C until needed. Each sample was then resuspended in 200µl ddH_2O, further diluted 1:10 and 1:100, and 10µl of each dilution was used as templates for amplification. Partial cytochrome *b* gene sequences were amplified with ERY11F and ERY12R primers. The PCR conditions and components were as described previously. Upon gel electrophoresis analysis, a PCR product of the expected size (~500bp) was found. This product was cloned in the TA pCR2.1 vector and sequenced as described previously. Upon sequence analysis, it was found that the cytochrome *b* sequence amplified from *E. graminis* f.sp. *hordei* was identical to that from *E. graminis* f.sp. *tritici* apart from 1bp (T to A change) 379bp downstream of the second base of the G_{143} codon. This base change would not lead to an amino acid change in the final protein (i.e. a silent mutation).

31 different *E. graminis* isolates were studied using the above protocol and in all cases the sequence found was consistent with the $G_{143}A$ mutation being the cause for resistance to strobilurin analogues in *E. graminis* f.sp. *tritici*. The A_{143} resistance allele was not detected in the *E. graminis* f.sp. *hordei* samples.

Specific ARMS *E. graminis* primers were designed on the basis of the above information on the $G_{143}A$ point mutation:

a forward ARMS primer based on the wild type sequence:

G-sp-1: CCATACGGGCAGATGAGCCACTGGAG (SEQ ID NO 126)

a forward ARMS primer based on the $G_{143}A$ mutation:

C-sp-1: CCATACGGGCAGATGAGCCACTGG**AC** (SEQ ID NO 127)

and a control primer designed upstream from the point mutation:

STAND2: GCCATACGGGCAGATGAGCCACTG (SEQ ID NO 128)

In both the G-sp-1 and the C-sp-1 primers, the -1 base (the 3'end base) corresponds to the second nucleotide of the G_{143}/A_{143} codon. Bases in the primers that differ from the wild type cytochrome *b E. graminis* sequence are in bold. The -2 position was changed from a G to a A base. This was done to destabilise the primer, as is normal in ARMS reactions.

The Scorpion™ product detection system was used in this case as a detection mechanism. Again the Scorpion primer was designed using Oligo 5 and the MFold (see details in Example1) programs. The sequence of the *E. graminis* Scorpion primer was:

5' FAM-CCCGCC**GTTTTAGCTGCTTTAGCTTTAATGC**GGCGGG (SEQ ID NO 129)

MR-HEG-*AACACCTAAAGGATTACCAGATCCTGCAC* 3' (SEQ ID NO 130)

Underlined regions are the hairpin forming parts, FAM is the fluorescein dye, MR is a non-fluorogenic quencher attached to a uracil residue and HEG is the replication blocking hexethylene glycol monomer. The sequence in italics is the reverse primer sequence and the sequence in bold is the Scorpion sequence which anneals to the extension product of the reverse primer.

All primers were synthesised by Oswel DNA service. Before use, the primers were diluted to 5µM in a total volume of 500µl each. They were then further diluted to a final concentration of 500nM in the PCRs.

Primers were first validated for use in ARMS/Scorpion analyses by using plasmid DNA and total DNA as templates. This was performed in order to check the specificity and sensitivity of the primer designs. DNA fragments comprising partial wild type cytochrome *b* gene sequence and the corresponding sequence containing the $G_{143}A$ mutation were cloned in the TA pCR2.1 vector to be used in this validation process. Plasmid DNA was always diluted in 1mg/ml BSA prior to use as template in real time PCR assays. Fungal DNA for analysis was extracted from an *E. graminis* f.sp. *tritici* strobilurin analogue-sensitive control isolate using a phenol/chloroform extraction method (as described previously). A conidial *E. graminis* f.sp. *tritici* sample from a French strobilurin analogue-sensitive isolate (F12C) and a conidial *E. graminis* f.sp. *tritici* sample from a German strobilurin analogue-resistant isolate (11-8) were then tested using the validated primers at two conidial dilutions. All three fungal isolates originated from single pustules.

In all cases AmpliTaq Gold enzyme (Perkin-Elmer/ABI) was included in the reaction mix at 1unit/25µl reaction. The reaction mix also contained 1x buffer (10mM Tris-HCl (pH8.3), 50mM KCl, 3.5 mM $MgCl_2$, 0.01% gelatine), 100µM dNTPs. Amplifications were performed in an ABI Prism 7700 instrument for continuous fluorescence monitoring. A preliminary incubation of 95°C for 10minutes was performed followed by 50 cycles of 95°C for 15sec and 60°C for 45sec. Fluorescence was monitored during the annealing/extension stage throughout all cycles.

When tested against the control templates (plasmids and DNA from the strobilurin analogue-sensitive control isolate), the *E. graminis* ARMS/Scorpion primers showed good specificity with no evidence of mispriming occurring from the wrong template. In Figure 3a, amplification of the strobilurin analogue-sensitive control *E.graminis* DNA at a 1:100 dilution with the three primer mixes (Stand 2 + *E. graminis* Scorpio , G-sp-1 + *E. graminis*

Scorpio and C-sp-1 + *E. graminis* Scorpio) are shown. Each reaction was carried out in duplicate. Control and G specific primer reactions emit a strong fluorescence signal whilst the C specific primer reaction does not show any increase in fluorescence. The control and G-specific ARMS primers have recognised and bound to the template whilst the C-specific primer did not bind to the template present in the reaction. In this case, the G_{143}:A_{143} allele analysis is indicating only presence of the wild type allele.

Figure 3b illustrates PCRs where the French sensitive isolate (F12C) was analysed with the three primer mixes (Stand 2 + *E. graminis* Scorpion, G-sp-1 + *E. graminis* Scorpion and C-sp-1 + *E. graminis* Scorpion). In each case ~200mg conidia were suspended in 200µl ddH_2O and diluted 1:100 and 1:1000 in ddH_2O. 5µl of the dilutions was added to the appropriate PCRs. On analysis again, the control and G-specific primer reactions emitted a strong signal whilst the C-specific primer reaction did not show any increase in fluorescence. This indicates that only the wild type allele has been detected in this sample. There is a definite delay in fluorescence being produced when using conidia as template for the reaction compared with using plasmid DNA as template. This is either due to the reduced copies of molecules that can be used as template being present in the reaction or due to inhibitory components being present in the conidial sample.

Figure 4 illustrates PCRs where the German resistant isolate at two conidial dilutions was amplified using the three primer mixes (Stand 2 + *E. graminis* Scorpio, G-sp-1 + *E. graminis* Scorpio and C-sp-1 + *E. graminis* Scorpio). In this case control and C-specific primer reactions emit strong signals whilst the G-specific primer reaction does not show any fluorescence. This indicates that only the mutant G_{143}A allele has been detected in this sample.

EXAMPLE 3

In Example 3, we report the characterisation of partial cytochrome *b Rhynchosporium secalis* gene sequence and a real time PCR study where various *R. secalis* isolates were screened for the G_{143}A resistance allele. This is an example where the G_{143}A assay was carried out on a species where the point mutation had not previously been identified.

Wild type isolates of *R. secalis* were collected from the UK and France (see Table 8: *R. secalis* isolate details). The K1124 and K3327 isolates could be considered "baseline" (collected prior to use of strobilurin analogues in the field) and the other isolates were obtained from trial sites which had been exposed to several sprays of strobilurin analogues

over a number of seasons. Infected barley leaves were hand picked and stored in a polythene bag and sent to Jealott's Hill Research Station (Zeneca Agrochemicals). Upon arrival in the laboratory single lesions were excised from the leaves, surface sterilised in ethanol (30 seconds) followed by a 0.1% sodium hypochlorite solution wash (2 minutes) then placed onto Lima Bean agar (see Table 9) and incubated under alternating 12 hour black light (365nm Philips TLD 18W/08 - Philips Lighting Ltd, Croydon, UK)/12 hour no light cycle at a constant temperature of 19°C for 4-5 days. Colonies growing out of the lesions were subcultured as an uncounted spore suspension on Lima Bean agar and incubated as above for approximately 7 days until sporulation was obtained. Resulting spores were removed and stored in liquid nitrogen until isolates were required. Retrieval of isolates was achieved by plating out the spore suspension onto Lima Bean agar and incubating as above for approximately 7 days until sporulation was obtained.

Isolate code	Country of origin
K1124	UK
K3327	UK
K3274	UK
K3276	UK
K3278	UK

Table 8: *R. secalis* isolate details

Partial cytochrome *b* gene sequence was obtained from two *R. secalis* isolates (K1124 and K3327). These isolates were grown from a suspension of 100,000 spores per ml inoculated in a medium with a non-fermentable carbon source shaking at 85rpm for 21 days at 19°C (12hrs light/12hrs dark) and the mycelia were collected by filtering through Miracloth and frozen at -20°C until required. DNA was produced from the mycelia by using a phenol/chloroform extraction protocol (see Example1). DNA yield and quality were checked by gel electrophoresis and then a serial stock dilution was made (1:10, 1:100 and 1:1000 in ddH_2O) for use as templates in PCR amplifications. PCRs were set up as described in earlier examples. The primers used were either degenerate primers (deg4F (5' AGGTYTRTAYTRYGGDTCWTA 3') (SEQ ID NO 131) and deg3R (5' AGCDATAACWCCTAATAATTT 3') (SEQ ID NO 132) designed from homologous regions of cytochrome *b* fungal genes (delineating the sequence encoding amino acid region 100-294

according to *S.cerevisiae* coding system) or *E. graminis* specific primers ERY11F and ERY12R (details as in Example 2). A band of the expected size (~500bp) was amplified using both primer pairs from each isolate and each PCR product was cloned in the pCR2.1 TA vector (Invitrogen). Wizard minipreps were carried out to identify clones with inserts of the expected size (~500bp) and 5 clones were submitted for sequencing from each cloning event using the M13 forward and reverse primers. Upon analysis with the relevant bioinformatics software, it was found that a novel cytochrome *b* gene sequence had been identified which was closely related to other ascomycete cytochrome *b* gene sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in Table 3.

Different specific ARMS *R. secalis* primers were designed around the $G_{143}A$ point mutation location:

two forward ARMS primer based on the wild type sequence:

G-sp-2: CCTTATGGACAGATGTCTTTATGA**T**G (SEQ ID NO 133)

G-sp-3: CCTTATGGACAGATGTCTTTATGA**A**G (SEQ ID NO 134)

two forward ARMS primer based on the predicted $G_{143}A$ mutation:

C-sp-2: CCTTATGGACAGATGTCTTTATGA**TC** (SEQ ID NO 135)

C-sp-3: CCTTATGGACAGATGTCTTTATGA**AC** (SEQ ID NO 136)

and a control primer designed upstream from the point mutation:

STAND3: TCCTTATGGACAGATGTCTTTATG (SEQ ID NO 137)

In the ARMS primers, the -1 base (the 3'end base) corresponds to the second nucleotide of the G_{143}/A_{143} codon. Bases in the primers which differ from the wild type cytochrome *b R. secalis* sequence are in bold. The -2 position was changed from a G to an A or T base. This was done to destabilise the primer, as is normal for ARMS primers.

The Scorpion™ product detection system was used in this case as a detection mechanism and the Scorpion reverse primer was again designed using the Oligo 5 and the MFold programs (details as in Example 1). The *R. secalis* Scorpion primer sequence was:

5' FAM-CCCGCC**ATATTAGCTGCATTAGTATTAATGC**GGCGGG (SEQ ID NO 138) -

MR-HEG-*TACACCTAAAGGATTACCTGACCCTGCAC* 3'(SEQ ID NO 139)

(See previous examples for details).

All primers were synthesised by Oswel DNA Service. Before use, the primers were diluted to 5µM in a total volume of 500µl each. The primers were then further diluted to a final concentration of 500nM in the PCRs.

Again primers were first validated for use in ARMS/Scorpion analyses by using plasmid DNA as template at various concentrations. This was performed in order to check the specificity and sensitivity of the primer designs. Partial wild type cytochrome *b* gene sequence and a corresponding sequence containing the $G_{143}A$ mutation were cloned in the TA pCR2.1 vector to be used in this validation process. As this mutation has not yet been found in *R. secalis* DNA, the A_{143} point mutation was incorporated into the sequence using a site directed mutagenesis strategy: the point mutation was incorporated into a primer design and this primer was used to amplify the region of interest using the wild type clone as template. PCRs were set up using standard methods as previously described and 30 cycles of 94°C for 45 sec, 56°C for 45 sec and 72°C for 1 min 30 sec were performed on a Hybaid Omn-E instrument. An initial incubation of 3 minutes at 94°C and a final extension incubation of 10 minutes at 72°C were also included. The ~370bp PCR product was cloned into the TA pCR2.1 vector and resulting clones were sequenced to check for any PCR induced errors prior to use in this experiment.

Undiluted, the plasmid DNA preparations were calculated to be at concentrations approx. $2x10^{11}$ molecules per µl. The two plasmids stocks were therefore diluted to $2x10^{7}$, 10^{5}, 10^{3} and 10^{1} molecules/µl in 1mg/ml BSA and 5µl aliquots were used of each dilution resulting in ~ $1x10^{8}$, 10^{6}, 10^{4} and 10^{2} molecules of plasmid in the respective PCRs. The PCR conditions and components were as described previously. In figure 5a, which shows a serial dilution of the G plasmid detected with the G primer mix, the detection of fluorescence is delayed by ~ 4 cycles with each 10 fold plasmid dilution. Figure 5b shows the highest concentration G (wild type (wt)) and C (mutant) cassette amplified with the G-sp-2 primer mix. The G primer does not misprime off the C template until very late in the PCR even though the DNA template concentration is high (~10^{8} molecules of template in reaction). The significant delay before the G mispriming event therefore demonstrates that the primer has a good window of specificity. The C-sp-2 primer set also shows good specificity through the specific and non specific plasmid dilutions (figure 6a and 6b). G-sp-2 and C-sp-2 primer mixes were used in following experiments instead of G-sp-3 and C-sp-3 primer mixes.

The second part of this study was to compare using total (genomic) DNA and cDNA as template for the PCR. Total DNA material was prepared from the *R.secalis* isolates using a phenol-chloroform extraction method (as described previously). Total RNA was extracted from 100mg of ground mycelia using the RNeasy Plant minikit (Qiagen catalogue No. 74903) according to the manufacturer's recommendation. First strand cDNA synthesis was then undertaken with 1µg of total RNA using RT PCR with the Advantage RT-PCR kit (Clontech catalogue No. K1402-1) according to the manufacturer's recommendation. Pools of total DNA and cDNA from three isolates (K3278, K3274 and K3276) were prepared. The total DNA pool was used as template diluted 1:100, 1:1000 and 1:10000 and the cDNA was used as template neat and diluted 1:5 and 1:10. In each case, 5µl of template was added to the PCRs. Real time PCR conditions described in Example 1 and 2 were also used except that in this case 40 cycles PCR were performed instead of 50. Figure 7a, b and c illustrate results obtained with total DNA and cDNA templates at three dilutions (dilution 1: total DNA (1:100) and cDNA (neat); dilution 2: total DNA (1:1000) and cDNA (1:5); dilution 3: total DNA (1:10000) and cDNA (1:10)) amplified using the G primer mix. Figures 8a, b and c illustrate the total DNA and cDNA templates amplified using the C primer mix. Using total DNA instead of cDNA as template gave more sensitive results with earlier Cycle threshold values. In order to give the best chance of detecting any C mutation, total DNA inputs at dilutions of 1:10 and 1:1000 were chosen for future analyses. No fluorescence changes could be detected when the C specific primer mix was used in this 40 cycles PCR indicating that only the wild type G_{143} allele was found in these isolates.

EXAMPLE 4

In Example 4, we report the characterisation of partial *Pyrenophora teres* cytochrome *b* gene sequence and a study where a $G_{143}A$ resistance allele detection assay was carried out on a variety of *P. teres* isolates. This is a species where the $G_{143}A$ mutation had not been identified previously. This example is divided into two sections; one describing a real time PCR study using an intercalating dye detection method using cDNA preparations of *P. teres* isolates and the other describing a real time PCR study using a Scorpion detection assay on genomic DNA preparations of *P. teres* isolates.

Real time PCR study using an intercalating dye:

Wild type isolates of *P. teres* (causal agent of barley net blotch) were collected from the UK and France during 1994, 1996 and 1998 (see Table 10 for *P. teres* isolate details).

1994 and 1996 isolates could be considered "baseline" (collected prior to use of strobilurin analogues in the field) and 1998 isolates were obtained from trial sites which had been exposed to several sprays of strobilurin analogues over a number of seasons. Infected barley leaves were hand picked and sent to Jealott's Hill Research Station (Zeneca Agrochemicals). Upon arrival in the laboratory, the leaves were incubated in a humid environment at 21° for 24-48 hours. Single lesions were excised from the leaves, surface sterilised in ethanol (30 seconds) followed by a 0.1% sodium hypochlorite solution wash (2 minutes) then placed onto Rose Bengal agar (see Table 9) and incubated under an alternating 12 hour black light/12 hour no light regime at a constant temperature of 22°C for 4-5 days. All media described in Table 9 were sterilised by autoclaving at 121 degrees C for 15 mins at 15psi. Colonies growing out of the lesions were subcultured by mycelial plug onto V8+streptomycin agar (see Table 9) and incubated as above for 14 days.

Media:	Ingredients:	
Potato Dextrose broth:	Potato Dextrose (Difco) Deionised water	24g 1000mls
Lima Bean Agar:	Lima Bean agar (Difco) Agar No. 3 (Oxoid) Deionised water	23g 10g 1000mls
Rose Bengal Agar:	Glucose (Oxoid) Yeast Extract (Oxoid) Agar No. 3 (Oxoid) Deionised water After autoclaving, add; Streptomycin sulphate (Sigma) Rose Bengal (Sigma)	10g 2g 17g 1000mls 125mg 25mg
V8 + streptomycin agar	V8 juice (Campbells) Calcium carbonate (Fisher) Agar No. 3 (Oxoid) Deionised water After autoclaving, add: Streptomycin sulphate (Sigma)	200mls 3g 20g 800mls 200mg

Czapek Dox V8 Agar	V8 juice (Campbells) Calcium carbonate (Fisher) Czapek Dox agar (Oxoid) No. 3 agar (Oxoid) Deionised water	200mls 3g 45.5g 7g 800mls
Suppliers:	Campbells Groceries, Kings Lynn, Norfolk, UK Oxoid, Basingstoke, UK Sigma Chemicals, Poole, UK Difco, Detroit, Michigan, USA Fisher Scientific, Loughborough, UK	

Table 9 Media recipes

Resulting mycelial and spore material was removed and stored in liquid nitrogen until isolates were required. Retrieval of isolates was achieved by plating out stored fungal samples onto V8 agar and incubating as above for 14 days until sporulation was obtained.

Isolate code	Year collected	Country of origin
K1056	1980	Ireland
K1916	1994	UK
K2346	1996	France
K2383	1996	UK
K2385	1996	UK
K2390	1996	UK
K2396	1996	UK
K3230	1998	UK
K3237	1998	UK
K3238	1998	UK
K3253	1998	UK

Table 10: *P. teres* isolate details

Partial cytochrome *b* sequence was identified from two isolates (K1056 and K1916). The initial wild type sequence was obtained from a biological sample prepared by inoculating potato dextrose broth (see Table 9) with a macerated mycelial suspension. The flask was incubated at 85rpm on an orbital shaker under a 12 hours white light/12 hours no light regime at a constant temperature of 19°C for 21 days until there was sufficient mycelial material for the DNA extraction protocol. The mycelium was then harvested by filtering through Miracloth and was frozen at -20°C until needed. Total DNA was produced using the phenol/chloroform extraction protocol (see Example 1). After the DNA yield and quality were checked by gel electrophoresis, a serial dilution was made (1:10, 1:100 and 1:1000 in ddH_2O) for use as PCR templates. Many different primer combinations were tried (specific and degenerates) and in most cases no amplification product was obtained. However, a primer pair that was designed to conserved *Aspergillus niger* and *Neurospora crassa*

cytochrome *b* sequences (Cytb3F and Cyt9R, see Example 2 for details) did give a ~500bp PCR product. This product was cloned in the TA pCR2.1 vector (Invitrogen) and 6 clones were sequenced using M13 forward and reverse primers. Upon analysis of the results, it was discovered that the reverse primer had correctly bound to cytochrome *b* sequence whilst the forward primer had misprimed in DNA sequence with intron-like features. A specific *P. teres* primer (Pt2R: 5' CTT ACA TCT GTA ATA GGT AAT 3') (SEQ ID NO 140) was designed in the novel stretch of cytochrome *b* gene and was used with a forward primer that was designed on the basis of *Venturia inaequalis* cytochrome *b* gene sequence (Pt5F: 5' TGT TAC TTT AGC AAT GCA CTA 3') (SEQ ID NO 141) on *P. teres* cDNA template. cDNA was produced from mycelial samples of the two isolates. Total RNA was extracted from 100mg of ground mycelium using the RNeasy kit (Qiagen) according to the manufacturer's recommendation. First strand cDNA synthesis was prepared from 1µg of total RNA using RT PCR with the Advantage RT-PCR Clontech kit (according to the manufacturer's recommendation). 5µl of the resulting cDNA was then used in PCRs. The PCR components and conditions were as described previously. A ~800bp PCR product was amplified for both isolates (delineating the sequence encoding amino acid region 48 to 310 of fungal cytochrome *b* according to the *S. cerevisiae* numbering system). In both cases, the PCR product was cloned in the TA pCR2.1 vector (Invitrogen) and 4 clones which contained an expected size insert (~800bp) were sequenced using M13 forward and reverse primers for each isolate. Sequence data analysis revealed that a novel cytochrome *b* gene sequence had been isolated that was closely related to other known ascomycete cytochrome *b* sequences. A 61 nucleotide tract of cDNA sequence encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in Table 3.

Specific ARMS *P. teres* primers were designed around the $G_{143}A$ point mutation location:

a forward ARMS primer based on the wild type sequence:

G-sp-4: CCCTACGGGCAAATGAGCCTTTGAAG (SEQ ID NO 142)

a forward ARMS primer based on the potential $G_{143}A$ mutation:

C-sp-5: CCCTACGGGCAAATGAGCCTTTGATC (SEQ ID NO 143)

and a control primer designed upstream from the point mutation:

STAND4: ACCCTACGGGCAAATGAGCCTTTG (SEQ ID NO 144)

the reverse primer used was:

UNLS4: TACACCTAAAGGATTTCCTGACCCTGCAA (SEQ ID NO 145)

Again in the ARMS primers, the -1 base (the 3'end base) corresponds to the second nucleotide of the G_{143}/A_{143} codon. Bases in the primers which differ from the wild type cytochrome *b P. teres* sequence are in bold. The -2 position was changed from a G to an A or T base. This was done to destabilise the primer.

All primers were synthesised by Oswel DNA Service. Before use, the primers were diluted to 5µM in a total volume of 500µl ddH_2O each. The primers were then further diluted to a final concentration of 500nM in the PCRs.

In this case, an intercalating dye was used for detection of the PCR product. The dye used was YO-PRO-1 dye (Molecular Probes, Seattle Washington, USA) which binds to double stranded DNA and emits fluorescence which is detectable by the ABI Prism 7700 instrument.

Primers were first validated by using plasmid DNA as template at various concentrations. This was done to check the specificity and sensitivity of the primer designs. Partial wild type *P. teres* cytochrome *b* gene sequence and a corresponding sequence containing the $G_{143}A$ mutation introduced by site directed mutagenesis were cloned in the TA pCR2.1 vector to be used in this validation process (as described in Example 3).

Undiluted, the plasmid DNA stocks were calculated to be approx. $2x10^{11}$ molecules per µl. The two plasmids were diluted to $2x10^7$, 10^5, 10^3 and 10^1 molecules/µl and 5µl were used of each dilution resulting in ~ $1x10^8$, 10^6, 10^4 and 10^2 molecules of plasmid in the respective PCRs (see Example 1 for PCR conditions). The only difference was that YO-PRO-1 dye was added to the reaction mix. The generation of primer dimer product interfered with the signal past cycle 35. This method of detection is less sensitive than the Scorpion detection system because it is more affected by background noise (e.g. fluorescence emission from primer dimer formation). Nevertheless, it was concluded that valuable information could be drawn from using this method but increased caution had to be taken in interpreting results.

In the following experiment, the isolates described above were checked for the presence of the $G_{143}A$ allele. Isolates were grown in a medium with a non-fermentable carbon source to obtain material for ARMS/Scorpion assays. An initial spore suspension was inoculated (1ml at 100,000 spores/ml) into conical flasks containing broth. The cultures were

incubated on an orbital shaker as previously described and the resulting mycelia were then harvested for use in cDNA preparations. cDNA material was prepared (as described previously) from each of the isolates. This was done to avoid designing primers within the complex intron/exon organisation of the *P. teres* cytochrome *b* sequence.

All PCRs were set up as previously described and 50 cycles were carried out. The only difference was that YO-PRO-1 dye was added to the reaction mix. cDNA neat and diluted 1:10 were used as template, in all cases 5µl of template were added to the PCR assays.

Figures 9 a and b illustrate the PCR amplification results of two of the eleven isolates tested in two dilutions, in duplicate with the three primer pairs. In no case were detectable levels of the $G_{143}A$ allele present in the samples tested.

Real time PCR study using a Scorpion detection system:

A diagnostic $G_{143}A$ Scorpion assay was also designed based on the genomic organisation that was determined for the *P. teres* cytochrome *b* gene encoding the amino acid region of interest.

The intron/exon organisation around the base of interest was elucidated by carrying out PCR amplifications on genomic DNA preparations with a series of primers designed to the known coding sequence and to subsequently identified intron sequences. Taq Extender™ PCR Additive and Perfect Match® PCR Enhancer (both from Strategene catalogue No. 600148 and 600129 respectively) were used as per manufacturer's instructions in the amplification of large PCR fragments using a variety of different primer combinations. After an initial 94°C incubation for 3 minutes, 30 cycles of 94°C for 45 sec, 52°C for 45 sec and 72°C for 3 minutes were carried out on a Hybaid Omn-E PCR instrument. A final 72°C incubation for 10 minutes was also included. When the PCR products were analysed using gel electrophoresis, a 2.7kb PCR product was found with the primer pair pter23F (coding region 5' ACA TAG TAA TAC TGC TTC AGC 3') (SEQ ID NO 146) - pter25R (intron region 5' TAC ATT TGA GGC AAA TAT TTC (SEQ ID NO 147) 3') and a 7.5kb PCR product was found with primer pair pter7F (coding region 5' CTA CGG GCA AAT GAG CCT TTG 3') (SEQ ID NO 148) - pter6R (coding region 5' CTC TGG AAC TAT CGC TGC AGG 3') (SEQ ID NO 149). These PCR products were cloned in the pCR-XL-TOPO vector (Invitrogen Catalogue No. K4700-10) according to the manufacturer's instructions and 2 clones for each cloning event were sequenced as described previously. The second base of the G_{143} codon

was found to be located at the 3' end of a 36bp exon. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in Table 3.

A series of 31bp ARMS primers and a control primer (for the amplification of both mutant and wild type alleles) were designed on the forward strand whilst the common Scorpion primer was designed on the reverse strand in intron sequence. The resulting amplicon was 121bp long (with the ARMS primers) and 123bp long (with the control primer). In this case, the second base of the G_{143} codon is on a 36bp exon, only 1bp away from the 3' splicing site. Again the Scorpion primer was designed using the Oligo 5 and MFold programs (see details in Example 1). The *P. teres* Scorpion primer sequence was:

5'FAM CCC GCC **GCA AGC TGA TTT CAT A**GG CGG G (SEQ ID NO 150) MR-HEG *TT CAA GTA CAT CCA ATT TCA ATA TAC ACT* 3' (SEQ ID NO 151)

The Scorpion primer description, the primer synthesis and real time PCR conditions were as described in previous examples.

In the optimisation of this Scorpion assay, a wider variety of primers than in previous Examples were tested for specificity, each with different mismatched bases at their 3' end to cause destabilisation (highlighted in bold), as shown below.

Primer	Sequence
STAND6	A CCC TAC GGG CAA ATG AGC CTT TGA G (SEQ ID NO 152)
PT-G-1	CCC TAC GGG CAA ATG AGC CTT TGA **AG** (SEQ ID NO 153)
PT-C-1	CCC TAC GGG CAA ATG AGC CTT TGA **AC** (SEQ ID NO 154)
PT-G-2	CCC TAC GGG CAA ATG AGC CTT TGA **CG** (SEQ ID NO 155)
PT-C-2	CCC TAC GGG CAA ATG AGC CTT TGA **CC** (SEQ ID NO 156)
PT-G-3	CCC TAC GGG CAA ATG AGC CTT **C**GA **AG** (SEQ ID NO 157)
PT-C-3	CCC TAC GGG CAA ATG AGC CTT **C**GA **AC** (SEQ ID NO 158)
PT-G-4	CCC TAC GGG CAA ATG AGC CTT **T**TA **CG** (SEQ ID NO 159)
PT-C-4	CCC TAC GGG CAA ATG AGC CTT **T**TA **CC** (SEQ ID NO 160)
PT-G-5	CCC TAC GGG CAA ATG AGC CTT **T**GC **GG** (SEQ ID NO 161)
PT-C-5	CCC TAC GGG CAA ATG AGC CTT **T**GC **GC** (SEQ ID NO 163)

Table 11: *P. teres* ARMS primers

Initial validation of the primers was carried out on the wild type and mutant plasmid template at $1x10^7$ molecules per reaction. Real time PCR on the ABI Prism 7700 instrument was carried out in duplicate for each of the plasmid templates, using both the G and C-

specific ARMS primers and the control primer (which amplifies both mutant and wild type alleles) with the common reverse Scorpion primer.

As seen in the Table 12, the different ARMS primer designs gave different windows of specificity when tested against mutant and wild type plasmid templates.

When compared to the other primers tested, PT-G-1 and PT-C-5 provided the widest window of specificity. They were therefore chosen as the preferred primer pair for this assay. Primer PT-C-5 has a Ct value of 16 on the correct template, and PT-G-1 also has a Ct value of 16 on the correct template. The PT-C-5 primer misprimes on the wrong template at cycle 34 giving a window of specificity of 18 cycles and the PT-G-1 primer misprimes at cycle 32 giving a window of specificity of 16 cycles equating to approximately 10 fold difference in the sensitivity of the two primers.

Primer	Ct on correct template	Ct on incorrect template	Window of specificity (cycles)
PT-G-1	16	32	16
PT-G-2	23	38	15
PT-G-3	FAILED	FAILED	FAILED
PT-G-4	32	42	10
PT-G-5	22	36	14
PT-C-1	20	34	12
PT-C-2	20	34	12
PT-C-3	28	38	10
PT-C-4	34	38	4
PT-C-5	16	34	18
C-SP-5	18	34	18

Table 12: ARMS primer validation

The PT-G-1 and PT-C-5 ARMS primers were tested in a plasmid spiking experiment where the mutant plasmid was 'spiked' in a background of wild type plasmid at frequencies of 1:1, 1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000, all in 1mg/ml BSA. In all cases the total plasmid concentration of each frequency in the PCR assay was $1x10^7$ molecules/reaction. Real time PCR conditions and components were as described previously.

The resulting data is summarised in the table below:

PCR template	PT-C-5 Ct values
100% C template	18
1:1	19
1:10	22
1:100	26
1:1000	30
1:10000	34
1:100000	36
100% G template	36

Table 13: Spiking experiment results

As the C plasmid reduces in the wild type plasmid background, the fluorescence detection is delayed. With each 10 fold dilution of the C plasmid, there is a delay of 4 cycles in the detection of fluorescence. The PT-C-5 cycle threshold value on one mutant molecule in a background of 10,000 wild type molecules could be clearly seen but the 1:100,000 was not distinguishable as the PT-C-5 primer misprimes from the 100% wild type (G) cassette thus masking the lower C:G frequency. This indicates that the ARMS switch in this case will allow a frequency of ≤1:10,000 to be detected with confidence.

A series of *P. teres* samples gathered from Cork in Ireland (Ir 5-8, Ir 9-13, Ir 14-17, Ir 18-21, Ir 30-34) were tested using the newly optimised real time Scorpion PCR assay. These groups of 4 or 5 isolates were inoculated onto V8 agar plates and incubated until sporulation of the resulting colony was achieved.

Mycelia were harvested from the agar plates in 10ml sterile ddH_2O. The mycelial suspension was transferred to a 15ml Falcon tube and centrifuged at 3200rpm for 10 minutes. The water was removed and the mycelial mass was divided between two sterile Eppendorf tubes. The mycelia were then pelleted by centrifugation at 13,000rpm for 5 minutes. The supernatant was removed using a pipette and one of the mycelial pellets was used in the DNA extraction, the other was kept at -80°C until needed. DNA extraction was carried out using the Qiagen DNeasy Plant Mini Kit protocol for isolation of DNA from plant tissue, as described in the manufacturer's protocol. The DNA was diluted 10-fold and 100-fold for use in the assay and 5µl aliquots of DNA were used in each PCR assay. All isolates were tested

with each primer pair (PT-C-5, PT-G-1 and the control primer; each with the common reverse primer containing the Scorpion detection system) in triplicate and in two dilutions.

The results of the isolate screening using the 1:10 dilution of genomic DNA as template are shown below:

Isolates	Cycle threshold values		
	C	G	S
Ir5-8 (1)	-	20	20
Ir5-8 (2)	-	22	22
Ir5-8 (3)	34	20	20
Ir9-13 (1)	-	18	18
Ir9-13 (2)	-	20	20
Ir9-13 (3)	36	20	20
Ir14-17 (1)	36	18	18
Ir14-17 (2)	-	22	22
Ir14-17 (3)	36	18	18
Ir18-21 (1)	-	20	20
Ir18-21 (2)	-	20	20
Ir18-21 (3)	34	18	18
Ir30-34 (1)	-	20	20
Ir30-34 (2)	34	20	20
Ir30-34 (3)	-	19	19

Table 14 : *P. teres* real time PCR results

In most cases the PT-C-5 primer only shows a cycle threshold value (Ct) at or later than cycle 34 at which mispriming is shown to occur on plasmid template. In some cases the PT-C-5 primer does not misprime at all. These results demonstrate that there was no evidence of the $G_{143}A$ mutation being present in the samples studied.

EXAMPLE 5

In Example 5, we report the characterisation of partial cytochrome *b Uncinula necator* gene sequence. *U. necator* is the causal agent of vine powdery mildew.

Infected vine leaves and fruit were collected from trial sites and commercial vineyards in France and Italy during 1999 and sent to Jealott's Hill Research Station (Zeneca Agrochemicals). Following arrival in the laboratory, mycelium and spores were transferred using a small paintbrush to fresh surface-sterilised leaves detached from 6-7 leaf seedlings (var. Ohanez). *U. necator* from each collection site was treated as a separate population.

On receipt in the laboratory the inoculated leaves were placed in a constant temperature room at 21°C and incubated for 2-3 weeks. Once sporulating disease was detected, the infected leaves were used to inoculate 3-4 leaf vine seedlings sown directly into a plastic plant propagator. A compressed airline was used to blow conidia off source leaves

onto appropriate target leaves of up to 40 vine seedlings per isolate. The lid was replaced on the propagator, which was then incubated in a controlled environment growth room for 2 weeks. Conidia produced on these plants were collected and frozen at -80°C until needed.

Total RNA was extracted from 100mg of ground *U. necator* spores originating from one isolate using the RNeasy kit from Qiagen (according to the manufacturer's recommendation). First strand cDNA synthesis was from 1µg of total RNA using RT PCR with the Advantage RT-PCR Clontech kit (according to the manufacturer's recommendation). 5µl of cDNA was used as template for PCR amplification using ERY 11F (5'ATGAACAATTGGTACAGTAAT 3') (SEQ ID NO 163) and ERY 4R (5' AAATCTGTTAAAGGCATAGCC 3') (SEQ ID NO 164) which delineate amino acids 114 to 309 of fungal cytochrome *b* based on the *S. cerevisiae* numbering system. A DNA fragment of the expected size (~500bp) was amplified and the PCR product was cloned in the pCR2.1 TA vector (Invitrogen) according to manufacturer's recommendations. Wizard minipreps were carried out to identify clones with suitable inserts and 5 clones were submitted for sequencing using the M13 forward and reverse primers. Upon analysis with the relevant bioinformatics software (Seqman and Macaw), it was found that a novel cytochrome *b* gene sequence had been identified which was closely related to other ascomycete cytochrome *b* sequences. A 61 nucleotide tract of cDNA sequence encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in Table 3. Specific *U. necator* primers were designed based on the novel sequence for use directly on spore material. The presence of intron sequences within the region of interest (500bp around the G_{143} SNP) was suspected as no previous PCR amplification on biological material had been successful. Taq Extender™ PCR Additive and Perfect Match® PCR Enhancer (both from Strategene) were used (as per manufacturer's instructions) in the amplification of large PCR fragments using a variety of different primer combinations. After an initial 94°C incubation for 3 minutes, 30 cycles of 94°C for 45 sec, 52°C for 45 sec and 72°C for 3 minutes were carried out on a Hybaid Omn-E PCR instrument. A final 72°C incubation for 10 minutes was also included. Primer combinations 2F (5' GTT TTA CCC TAC GGG CAG ATG3') (SEQ ID NO 165) -5R(5' AAA GAA TCT GTT TAA GGT TGC 3') (SEQ ID NO 166), 2F6R (5' AAA CCA CCT CAA AGA AAC TCC 3') (SEQ ID NO 167) and 4F (5' CAT GAA TAG GAC AAG ATA TCG 3') (SEQ ID NO 168) -6R successfully amplified PCR products ranging from 1.6kb to 3kb in length. These

PCR products were cloned in the TA pCR2.1 vector (Invitrogen) and subseq
used for sequencing as described previously. A primer walking strategy was foll
clone corresponding to each of the different PCR products and the sequence analysi. .nese
three clones showed the presence of two introns (1.6kb and 1.1 kb in length) within the PCR
fragments. The second base of the G_{143} codon is 10bp upstream of an intron splicing site. A
41 nucleotide tract encoding 10 bases upstream and 30 bases downstream of the second base
of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found
in Table 3.

EXAMPLE 6:

In example 6 we report the characterisation of partial *Sphaerotheca fuliginea* cytochrome *b* gene sequence and a single nucleotide polymorphism that gives rise to resistance to strobilurin analogues and compounds in the same cross resistance group. *S. fuliginea* is the causal agent for cucurbit powdery mildew.

S. fuliginea infected cucumber and melon leaves were collected from the field and conidia were dry inoculated in the laboratory to fresh leaf material (cucumber and melon) using a small paintbrush. Monoconidial isolates were subcultured and tested *in planta* by 24 hour preventative discriminating dose assay (up to 100ppm doses, known to give a 100% control of wild type strains). Conidia from candidate resistant isolates were removed by aspiration by vacuum pump into a suitable container. Part of this sample was analysed using PCR analysis and the remainder was re-tested to confirm phenotypic resistance.

An RT-PCR strategy was followed (as described in Example 3) on a ~100mg conidial strobilurin analogue sensitive sample. The resulting cDNA was used as template in a PCR amplification reaction using primers Ery2F (5' TCACCTAGAACATTAACATGA 169) 3'(SEQ ID NO) and 4R (5' AAATCTGTTAAAGGCATAGCC 3'(SEQ ID NO 170)) which delineate amino acids 108 to 309 of fungal cytochrome *b* based on the *S. cerevisiae* numbering system. Other PCR components and conditions were as described previously. A PCR product of the expected size (~600bp) was found during gel electrophoresis analysis of the PCR products and this product was then cloned in the TA pCR2.1 vector (Invitrogen) and 5 clones with correct size inserts (~600bp) were sequenced using M13 forward and reverse primers as described previously. Upon analysis with the relevant bioinformatics software (Seqman and Macaw), it was found that a novel cytochrome *b* gene sequence had been identified which was closely related to other ascomycete cytochrome *b* sequences. Specific

Sphaerotheca fuliginea PCR primers were then designed for the amplification of the G_{143} region from genomic DNA. Primer pairs SF1 (5' TTCCCTTCGGTCAAATGTCGC 3') (SEQ ID NO 171) - SF8 (5' AAACCCCCTCAGAGAAACTCC 3') (SEQ ID NO 172) and SF1 - SF10 (5' GACCCCGCGCTATCATGTAAG 3') (SEQ ID NO 173) were used in PCR amplifications using spore samples resuspended in ddH_20 as template. The PCR components and conditions were as described in previous examples. The PCR products were analysed by gel electrophoresis and a ~2kb product was found with the SF1/8 primer pair and a ~2.1kb band was found with the SF1/10 primer pair. Both PCR products were cloned in the TA pCR2.1 cloning vector (Invitrogen) and 5 clones were sequenced as described previously. Two clones from both cloning events were fully sequenced using a primer walking strategy. When the sequence data was analysed using the relevant bioinformatics software, it was found that a 1917bp intron was present 9bp downstream from the second base of the G_{143} codon. The cDNA and the genomic (taking in account the intron/exon organisation) sequences of a 37 nucleotide tract encoding 6 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in Table 3.

Partial cytochrome *b* gene sequence was also amplified from conidial strobilurin analogue resistant samples. Conidial samples (<50mg) were resuspended in 200µl ddH_20 and diluted 1:10, 1:100 in ddH_20. 10µl of each dilution was used as template for PCR amplification using the SF1/SF8 specific *S. fuliginea* primers. Taq Extender™ PCR Additive and Perfect Match® PCR Enhancer (both from Strategene) were used (as per manufacturer's instructions) in the amplification of this large PCR fragment. After an initial 94°C incubation for 3 minutes, 30 cycles of 94°C for 45 sec, 50°C for 45 sec and 72°C for 5 minutes were carried out on a Hybaid Omn-E PCR instrument. A final 72°C incubation for 10 minutes was also included. The PCR products were analysed using gel electrophoresis analysis and an expected size band (~2kb) was found. This was cloned in the TA pCR2.1 vector (Invitrogen) and 10 clones which had the expected size insert were sequenced as described in previous examples. Analysis of the sequence data using suitable bioinformatics software (Seqman, Editseq and Macaw software) revealed a G-->C point mutation in the cytochrome *b* sequence when compared to wild type sequence in all 10 cases. This DNA point mutation leads to a single glycine to alanine change at position 143 (according to the *S.cerevisiae* amino acid coding system).

EXAMPLE 7:

In example 7 we report the characterisation of partial *Mycosphaerella fijiensis* var. *difformis* cytochrome *b* gene sequence. *M. fijiensis* is the causal agent of black sigatoka disease on banana.

Infected banana leaves were collected from the field and ascospores inoculated from leaves directly onto artificial media in a petri dish. Monoascosporic isolates were maintained on artificial media and prepared for PCR analysis by shake flask culture in a broth medium. Mycelia were collected through Miracloth and ground to a fine powder using an acid washed and autoclaved sterile pestle and mortar. 100mg of ground mycelia were used in a genomic DNA extraction and in a first strand cDNA synthesis (as described in previous examples). The genomic DNA was diluted 1:10, 1:100 and 1:1000 in ddH_2O prior to use as PCR template. 5µl of cDNA and 10µl of each genomic DNA dilution were used as template for PCR amplification using the degenerate primer pair as described in Example 3. The PCR conditions and components were as described in previous Examples. Upon gel electrophoresis analysis of the PCR products, an expected size band (~500bp) was found when cDNA was used as template and a larger band (~1.6kb) was found when genomic DNA was used as template. The two PCR products were cloned in the pCR2.1 TA vector (Invitrogen) and five resulting clones with the correct size inserts (~500bp and 1.6kb) were sequenced using the M13 forward and reverse primers. Upon analysis of the sequence data using the relevant bioinformatics software, it was found that the larger PCR product contained a 1064bp intron, 78bp downstream from the second base of the G_{143} codon. Apart from the presence of the intron in the larger PCR product, the DNA sequences were identical. This cytochrome *b* gene sequence was identified as novel and was closely related to other ascomycete cytochrome *b* sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S. cerevisiae* amino acid numbering system) can be found in Table 3.

EXAMPLE 8:

In example 8 we report the characterisation of partial *Pseudoperonospora cubensis* cytochrome *b* gene sequence. *P. cubensis* is the causal agent of cucurbit downy mildew.

Sporangia were washed off infected leaf material into deionised water. The resulting sporangial suspension was inoculated by spray gun onto fresh leaf material at a concentration

of 10,000 spores per ml. After disease had developed, a sample of spore suspension, collected in ddH_20, was spun in a centrifuge to produce a pellet and kept at -80°C until needed. The *P. cubensis* sporangia were then resuspended in 200µl of ddH_20 and diluted 1:10 and 1:100 in ddH_20. 10µl of each sporangia dilution was used as template for PCR amplification using primers PLAS17F and PLAS15R (see details in Example 1). The PCR conditions and components were as described in previous Examples. Upon gel electrophoresis analysis of the PCR products, an expected size band (~500bp) was found. This PCR product was then cloned in the pCR2.1 TA vector and five resulting clones with the correct size inserts (~500bp) were sequenced using the M13 forward and reverse primers. Upon analysis with the relevant bioinformatics software, it was found that a novel cytochrome *b* gene sequence had been identified which was closely related to other oomycete cytochrome *b* sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S. cerevisiae* amino acid numbering system) can be found in Table 3.

EXAMPLE 9:

In example 9 we report the characterisation of partial *Mycosphaerella graminicola* cytochrome *b* gene sequence. *M. graminicola* is the causal agent of leaf blotch on wheat.

Infected wheat leaves were collected from the field and incubated in a humid environment to promote spore production. Cirri were removed from leaves and spread onto Czapek Dox V8 agar plates (see Table 9) and incubated in a controlled environment at 19°C for 6 days. Single colony isolates were further subcultured and bulked up by shake flask culture in a suitable medium. Fungal material was removed and maintained at -80°C until needed. A genomic DNA preparation was carried out as described in Example 1 on 200mg of ground mycelia. The genomic DNA yield and quality was checked by gel electrophoresis and diluted 1:10 and 1:100 in ddH_20. 10µl of each dilution was used as template for PCR amplification using primers Cyt3F and Cyt9R (details in Example 2). PCR components and conditions were as described in Example 2. The PCR products were analysed by gel electrophoresis and a band of the expected size was found (~500bp). This PCR product was cloned in the TA pCR2.1 vector and 5 clones containing correct size inserts were sequenced as described before. When the sequencing data was analysed using the relevant bioinformatics software, the PCR fragment was found to encode part of a novel cytochrome *b* gene sequence which showed close homology to those of other ascomycete's. A 61

nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in Table 3.

EXAMPLE 10:

In example 10 we report the characterisation of partial *Colletotrichum graminicola* cytochrome *b* gene sequence. *C. graminicola* is the causal agent of cereal and grass anthracnose.

Infected leaf material (turf or moss) was collected from the field and the fungal material removed and subcultured on artificial media. Fungal material was bulked up by shake flask culture, harvested and kept at -80°C until needed. A genomic DNA preparation was carried out as described in Example 1 on 200mg of ground mycelia. The genomic DNA yield and quality was assessed by gel electrophoresis and dilutions of 1:10 and 1:100 in ddH_2O were prepared. 10µl of each dilution was used as template for PCR amplification using primers deg4F and deg3R (details in Example 3). PCR components and conditions were are described in Example 3. The PCR products were analysed by gel electrophoresis and a band of the expected size was found (~500bp). This PCR product was cloned in TA pCR2.1 vector and 5 clones containing correct size inserts were sequenced as described before. Upon analysis with the relevant bioinformatics software, it was found that a novel cytochrome *b* gene sequence had been identified and that this was closely related to other ascomycete cytochrome *b* sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in Table 3.

EXAMPLE 11:

In example 11 we report the characterisation of partial *Colletotrichum gloeosporioides* cytochrome *b* gene sequence. *C. gloeosporioides* is the causal agent of fruit anthracnose (e.g. pepper, avocado and mango).

Infected plant material (mango or chilli) was collected from the field and fungal material was removed and subcultured on artificial media. Mycelia was bulked up in shake flask culture and harvested by filtration through Miracloth. The mycelia were stored at -80°C until needed. The mycelia were ground using an acid washed sterile pestle and mortar and 100mg was used in a genomic DNA preparation and a first strand cDNA synthesis (procedures as described in previous examples). 10µl of each genomic dilutions and 5µl neat cDNA was

used as template for PCR amplification using primers deg4F and deg3R (primer details in Example 3). PCR components and conditions were as described in previous Examples. The PCR products were analysed by gel electrophoresis and bands of the expected size were found (~500bp). The PCR product was the same size when using genomic DNA or cDNA as template which indicates the lack of introns in the DNA region amplified. The PCR products were cloned in TA pCR2.1 vector and 5 clones containing correct size inserts for each cloning event were sequenced as described in previous Examples. When the sequencing data was analysed using the relevant bioinformatics software, the PCR products were found to be identical in all cases and to encode a novel cytochrome *b* gene sequence with close homology to other ascomycete sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in Table 3.

EXAMPLE 12:

In example 12 we report the characterisation of partial *Oidium lycopersicum* cytochrome *b* gene sequence. *O. lycopersicum* is a causal agent for tomato powdery mildew.

Diseased tomato leaves were collected from the field and conidia subcultured to fresh leaf material by dry inoculation in a settling tower. Conidia growing from the resulting infection were removed by aspiration by vacuum pump into a sterile Eppendorf tube and kept in at -80°C until needed. A first strand cDNA synthesis was carried out on RNA isolated from 100mg of spores as described previously and 5µl of this cDNA was used for PCR amplification using primers Ery2-4 (details in example 6). The PCR components and conditions were as described previously. When the PCR products were analysed by gel electrophoresis, a PCR product was found at the expected size (~500bp). This PCR product was cloned and 5 clones containing the correct size insert (~500bp) were sequenced as described previously. Upon analysis with the relevant bioinformatics software, it was found that the PCR fragment encodes a novel cytochrome *b* gene sequence which was closely related to other ascomycete cytochrome *b* sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in Table 3.

EXAMPLE 13:

In example 13 we report the characterisation of partial *Leveillula taurica* cytochrome *b* gene sequence. *L. taurica* is a causal agent for tomato powdery mildew.

Diseased pepper leaves were collected from the field and conidia dry inoculated to fresh leaf material on whole plants. Conidia from resulting infections were removed by aspiration by vacuum pump into a suitable container and analysed using PCR. Diseased pepper and tomato leaves were collected from the field and glasshouse at Jealott's Hill and infected leaf material was used directly in PCR analysis.

A first strand cDNA synthesis was carried out on RNA isolated from 100mg of spores or diseased plant material as described previously. When diseased plant material was used as starting material, fungal lesions were enriched by removing non-diseased plant tissue from the preparation. 5µl cDNA aliquots were used for PCR amplification using primers Ery11-12 (details in example 2). PCR components and conditions were as described previously. When the PCR products were analysed by gel electrophoresis, a PCR product was found at the expected size (~500bp). This PCR product was cloned and 5 clones containing the correct size insert (~500bp) were sequenced as described previously. Upon analysis with the relevant bioinformatics software, it was found that the PCR product encodes a novel cytochrome *b* sequence which was closely related to other ascomycete cytochrome *b* sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in Table 3.

EXAMPLE 14:

In example 14 we report the characterisation of partial *Alternaria solani* cytochrome *b* gene sequence. *A. solani* is the causal agent for early blight in tomato and potato. Infected leaf material (tomato or potato) was collected from the field and the fungal material removed and subcultured on artificial media. Fungal samples were bulked up in shake flask culture. Culture collection isolates had been stored in liquid nitrogen and periodically subcultured on artificial media or subcultured through live host material before re-storing at -80°C. Mycelia grown in a shake flask were ground using an acid washed sterile pestle and mortar and 100mg was used in a genomic DNA preparation and a first strand cDNA synthesis (procedures as described in previous examples). 10µl of each genomic dilutions and 5µl neat cDNA was used as template for PCR amplification using primers deg4F and deg3R (details in Example 3). PCR components and conditions were as described in

Example 3. The PCR products were analysed by gel electrophoresis and bands of the expected size were found (~500bp). The PCR product was the same size when using genomic DNA or cDNA as template which indicates the lack of introns in the DNA region amplified. The PCR products were cloned in TA pCR2.1 vector and 5 clones containing correct size inserts for each cloning event were sequenced as described before. When the sequencing data was analysed using the relevant bioinformatics software, the PCR products were found to be identical in all cases and to encode a novel cytochrome *b* gene sequence which shows close homology to other ascomycete sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S. cerevisiae* amino acid numbering system) can be found in Table 3.

A sequence only 12bp different from the previously isolated *A. solani* sequence was also amplified when using genomic DNA extracted directly from field infected tomato leaves as template for PCR amplification using primers Ery11-12 (see above for details). Although this DNA sequence contained 12 nucleotide differences, it did not result in any difference at the amino acid level when the two sequences were translated. The significant difference between these two sequences was the presence of an ~1.2kb intron, 62bp downstream of the second base in the G_{143} codon. These differences in intron/exon organisation and codon usage are evidence that such variations are possible within one species.

EXAMPLE 15:

In example 15 we report the characterisation of partial *Cercospora arachidola* cytochrome *b* gene sequence. *C. arachidola* is the causal agent of peanut leaf blotch.

Infected leaf material (peanut) was collected from the field and the fungal material removed and subcultured on artificial media. Cultures were stored in liquid nitrogen until required. Material was removed from storage onto agar and resulting colonies bulked up in shake flask culture and kept at -80°C until needed. Mycelia were ground using an acid washed sterile pestle and mortar and 100mg was used in a genomic DNA preparation and a first strand cDNA synthesis (procedures as described in previous examples). 10µl of each genomic dilutions and 5µl neat cDNA were used as template for PCR amplification using primers deg4F and deg3R (details in Example 3). PCR components and conditions were are described in Example 3. The PCR products were analysed by gel electrophoresis and bands of the expected size were found (~500bp). Again the PCR product was the same size when using genomic DNA or cDNA as template which indicates the lack of introns in the DNA

region amplified. The PCR products were cloned in TA pCR2.1 vector and 5 clones containing correct size inserts for each cloning event were sequenced as described before. When the sequencing data was analysed using the relevant bioinformatics software, the PCR products were was found to be identical in all cases and to encode a novel cytochrome *b* gene sequence which showed close homology to other cytochrome *b* gene sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in Table 3.

EXAMPLE 16:

In example 16 we report the characterisation of partial *Rhizoctonia solani* cytochrome *b* gene sequence. *R. solani* is the causal agent of root stem rot or damping off.

Infected leaf material (rice) was collected from the field and the fungal material removed and subcultured on artificial media. Cultures were stored in liquid nitrogen until required. Material was removed from storage onto agar and resulting colonies bulked up in shake flask culture and kept at -80°C until needed. A first strand cDNA synthesis was carried out on RNA isolated from 100mg of ground mycelia as described previously and 5µl was used for PCR amplification using basidiomycete degenerate primers 1F (5' WYTRGTAYTAATGATGGCTATHGG 3') (SEQ ID NO 174) and 1R (5' TCTTARWATWGCATAGAAWGG) 3' (SEQ ID NO 175) which delineate amino acids 121 to 283 of fungal cytochrome *b* according to the *S. cerevisae* numbering system. PCR components and conditions were as described previously. When the PCR products were analysed by gel electrophoresis, a PCR product was found at the expected size (~500bp). This PCR product was cloned and 5 clones containing the correct size insert (~500bp) were sequenced as described previously. When the sequencing data was analysed using the relevant bioinformatics software, the PCR product was found to encode a novel cytochrome *b* gene sequence which showed close homology to other basidiomycete sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in Table 3.

EXAMPLE 17:

In example 17 we report the characterisation of partial *Pythium aphanidermatum* cytochrome *b* gene sequence. *P. aphanidermatum* is the causal agent of damping off.

Infected leaf material (turf) was collected from the field and the fungal material removed and subcultured on artificial media. Cultures were stored in liquid nitrogen until required. Material was removed from storage onto agar and resulting colonies bulked up in shake flask culture and kept at -80°C until needed. A genomic DNA preparation was carried out as described in Example 1 on 200mg of ground mycelia. The genomic DNA yield was analysed by gel electrophoresis and stocks were diluted 1:10 and 1:100 in ddH_2O. 10µl of each dilution was used as template for PCR amplification using primers PLAS17F and PLAS15R (details in Example 1). PCR components and conditions were are described in Example 1. The PCR products were analysed by gel electrophoresis and a band of the correct size was found (~500bp). This PCR product was cloned in TA Invitrogen pCR2.1 vector and 5 clones containing correct size inserts were sequenced as described before. When the sequencing data was analysed using the relevant bioinformatics software, the PCR fragment was found to encode a novel cytochrome *b* gene sequence which showed close homology to other oomycete sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the *S.cerevisiae* amino acid numbering system) can be found in Table 3.

EXAMPLE 18

In example 18 we report the characterisation of partial *Mycosphaerella musicola* cytochrome *b* gene sequence. *M. musicola* is the causal agent of yellow sigatoka disease on banana.

Infected banana leaves were collected from the field and ascospores inoculated from leaves directly onto artificial media in a petri dish. Monoascosporic isolates were maintained on artificial media and prepared for PCR analysis by shake flask culture in a broth medium. Mycelia were collected through Miracloth and ground to a fine powder using an acid washed sterile pestle and mortar. 100mg of ground mycelia were used in a genomic DNA extraction and in a first strand cDNA synthesis (as described in Example 7). The genomic DNA was diluted 1:10, 1:100 and 1:1000 in ddH_2O prior to use as PCR template. 5µl of cDNA and 10µl of each genomic dilution were used as template for PCR amplification using the degenerate primer pair F4/R3 (as described in Example 3). The PCR conditions and components were as described in previous Examples. Upon gel electrophoresis analysis of

the PCR products, an expected size band (~500bp) was found when both the genomic DNA and cDNA was used as template. The two PCR products were cloned in the pCR2.1 TA vector and 5 resulting clones with the correct size inserts (~500bp) were sequenced using the M13 forward and reverse primers. Upon analysis of the sequence data using the relevant bioinformatics software, it was found that the PCR fragments encoded a novel cytochrome *b* gene sequence with close homology to other ascomycete cytochrome *b* gene sequences. A 61 nucleotide tract encoding 30 bases upstream and 30 bases downstream of the second base of the G_{143} codon (according to the amino acid numbering system) can be found in Table 3.

CLAIMS

1. A method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of said mutation in fungal nucleic acid using any (or a) single nucleotide polymorphism detection technique.

2. A method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to the presence or absence of said mutation in said nucleic acid.

3. A method for detecting a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group which method comprises contacting a test sample comprising fungal nucleic acid with an appropriate diagnostic primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended either when the said mutation is present in the sample or when wild type sequence is present; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

4. A method for detecting a mutation in fungal nucleic acid according to claim 2 wherein said method comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for the specific mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when the said mutation is present in the sample; and detecting the

presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

5. A method according to any of the preceding claims wherein the mutation is present in a fungal cytochrome *b* gene where said mutation results in the inhibition of a strobilurin analogue or any other compound in the same cross resistance group to the active site of the cytochrome b protein but still allows the respiration process to occur.

6. A method according to the previous claim wherein the mutation in the fungal nucleic acid results in the replacement of a glycine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the encoded protein with an amino acid selected from the group arginine, serine, cysteine, valine, aspartic acid and alanine.

7. A method according to any of the preceding claims wherein the mutation in the fungal nucleic acid results in the replacement of a glycine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the encoded protein with an alanine.

8. A method according to claim 2 for the detection of a mutation in a fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein wherein said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid with a primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to presence or absence of said mutation in said nucleic acid.

9. A method according to claim 3 for the detection of a mutation in a fungal cytochrome *b* gene resulting in a $G_{143}A$ replacement in the encoded protein said method comprising contacting a test sample comprising fungal nucleic acid with a diagnostic

primer for the mutation resulting in a $G_{143}A$ replacement in the encoded protein in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when a mutation is present in the sample resulting in a $G_{143}A$ replacement in the encoded protein; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

10. A method according to any of the preceding claims wherein the fungal gene is present in a plant pathogenic fungus selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporiodes, Oidium lycopersicum, Magnaporthe grisea, Phytophthora infestans, Leveillula taurica, Psuedoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.*

11. A method for detecting fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a single nucleotide polymorphism occurring at a position corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein.

12. A method according to claim 11 wherein the said single nucleotide polymorphism occurs at a position corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143.

13. A method according to any of the preceding claims wherein the single nucleotide polymorphism which is detected is a G to C base change occurring at a position

corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the cytochrome *b* protein.

14. A fungal DNA sequence encoding all or part of a cytochrome *b* protein wherein said DNA sequence encodes a glycine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 and is obtainable from a fungus slected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, , Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.*

15. A fungal DNA sequence according to claim 14 comprising all or part of a DNA sequence selected from the group SEQ ID NO 1; SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6, SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19, SEQ ID NO 20 and SEQ ID NO 21

16. A fungal DNA sequence encoding all or part of a cytochrome *b* protein which, when said sequence is lined up against the corresponding wild type DNA sequence encoding a cytochrome *b* protein, is seen to contain a single nucleotide polymorphism mutation at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein which results in the replacement of the normal glycine residue with an alternative amino acid with the proviso that said DNA sequence is not the *Mycena galopoda* sequence encoding cytochrome *b*.

17. A fungal DNA sequence according to claim 16 wherein said single nucleotide polymorphism mutation occurs at a position corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein which results in the replacement of the normal glycine residue with an alternative amino acid with the proviso that said DNA sequence is not the *Mycena galopoda* sequence encoding cytochrome *b*.

18. A fungal DNA sequence encoding all or part of a cytochrome *b* protein wherein said DNA sequence encodes an alanine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 and is obtainable from a fungus slected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea, Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola.*

19 A fungal DNA sequence encoding all or part of a cytochrome *b* protein according to claim 18 wherein said DNA sequence contains a single nucleotide polymorphism which results in the replacement of the normal guanine reside with a cytosine residue at a position corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein with the proviso that said DNA sequence is not the *Mycena galopoda* sequence encoding cytochrome *b*.

20. A fungal DNA sequence according to any of claims 16 to 19 comprising all or part of a sequence selected from the group SEQ ID NO 176; SEQ ID NO 177, SEQ ID NO 178, SEQ ID NO 179, SEQ ID NO 180, SEQ ID NO 181, SEQ ID NO 182, SEQ ID NO 183, SEQ ID NO 184, SEQ ID NO 185, SEQ ID NO 186, SEQ ID NO 187, SEQ ID NO 188, SEQ ID NO 189, SEQ ID NO 190, SEQ ID NO 191, SEQ ID NO 192, SEQ ID NO 193, SEQ ID NO 194, SEQ ID NO 195 and SEQ ID NO 196.

21. A fungal cytochrome *b* protein which confers fungal resistance to a strobilurin analogue or a compound within the same cross resistance group wherein in said protein a normal glycine residue is altered due to the presence of a mutation in the DNA coding for said protein said mutation occurring at a position corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein with the proviso that protein is not the *Mycena galopoda* cytochrome *b* protein.

22. A fungal cytochrome *b* protein according to claim 21 wherein in said protein a normal glycine residue is altered due to the presence of a mutation in the DNA coding for said protein said mutation occurring at a position corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the protein with the proviso that protein is not the *Mycena galopoda* cytochrome *b* protein.

23. A method for the detection of a mutation in fungal cytochrome *b* gene resulting in the replacement of a glycine residue in the encoded protein at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 said method comprising identifying the presence and absence of said mutation in a sample of fungal nucleic acid wherein any (or a) single nucleotide polymorphism detection method is based on the sequence information from around 30 to 90 nucleotides upstream and/or downstream of the position corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in either the wild type or mutant protein.

24. A method according to claim 23 wherein any (or a) single nucleotide polymorphism detection method is based on the sequence information from around 30 to 90 nucleotides upstream and/or downstream of the position corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in either the wild type or mutant protein.

25. An allele specific oligonucleotide capable of binding to a fungal nucleic acid sequence encoding a wild type cytochrome *b* protein selected from the group consisting of *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola* wherein said oligonucleotide comprises a sequence which recognises a nucelic acid sequence encoding a glycine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 .

26. An allele specific oligonucleotide capable of binding to a fungal nucleic acid sequence encoding a mutant cytochrome *b* protein wherein said oligonucleotide comprises a sequence which recognises a nucleic acid sequence encoding an amino acid selected from the group arginine, serine, cysteine, valine, aspartic acid, glutamic acid, tryptophan, or alanine.at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 .

27. A diagnostic primer or a diagnostic oligonucleotide capable of binding to a template comprising a mutant type fungal cytochrome *b* nucleotide sequence wherein the final 3' nucleotide of the primer or oligonucleotide corresponds to a nucleotide present in said mutant form of a fungal cytochrome *b* gene and the presence of said nucleotide gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group.

28. A diagnostic primer according to claim 27 wherein either the penultimate nucleotides (-2) or (-3) of the primer is not the same as that present in the corresponding position in the wild type cytochrome *b* sequence.

29. One or more diagnostic primers for detecting a $G_{143}A$ mutation in a fungal cytochrome *b* gene selected from the group consisting of SEQ ID NO 22, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 26, SEQ ID NO 27, SEQ ID NO 28, SEQ ID NO 29, SEQ ID NO 30, SEQ ID NO 31, SEQ ID NO 32, SEQ ID NO 33, SEQ ID NO 34, SEQ ID NO 35, SEQ ID NO 36, SEQ ID NO 37, SEQ ID NO 38, SEQ ID NO39, SEQ ID NO 40, SEQ ID NO41 and SEQ ID NO 42
and derivatives thereof wherein the final nucleotide at the 3' end is identical to the sequences given above and wherein up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides may be varied without significantly affecting the properties of the diagnostic primer.

30. An allele specific oligonucleotide probe capable of detecting a fungal cytochrome *b* gene polymorphism at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome b residue 143 in the protein.

31. An allele specific oligonucleotide probe according to claim 30 wherein said polymorphism is at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome b residue 143 in the protein.

32. An allele specific oligonucleotide probe according to claim 31 wherein the polymorphism is a guanine to cytosine base change.

33. A diagnostic kit comprising one or more of the diagnostic primers as claimed in claims 27 to 29, or an allele specific oligonucleotide as claimed in claims 25 or 26 or an allele specific oligonucleotide probe as claimed in claim 30 to 32, nucleotide triphosphates, polymerase, and buffer solution.

34. A method of detecting plant pathogenic fungal resistance to a fungicide said method comprising detecting a mutation in a fungal gene wherein the presence of said

mutation gives rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene using any (or a) single nucleotide polymorphism technique.

35. A method of detecting plant pathogenic fungal resistance to a fungicide said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises contacting a test sample comprising fungal nucleic acid with a primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to presence or absence of a mutation in said nucleic acid wherein the presence of said mutation gives rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene.

36. A method of detecting plant pathogenic fungal resistance to a fungicide according to claim 34 or claim 35 said method comprising contacting a test sample comprising fungal nucleic acid with a diagnostic primer for a specific mutation the presence of which gives rise to fungicide resistance in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

37. A method of detecting and quantifying the frequency of a mutation giving rise to plant pathogenic fungal resistance to a fungicide whose target protein is encoded by a mitochondrial gene said method comprising detecting the presence or absence of a mutation in a fungal gene wherein the presence of said mutation gives rise to fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying and quantifying the presence or absence of said mutation in fungal nucleic acid using any (or a) single nucleotide polymorphism detection technique.

38. A method according to claim 37 said method comprising detecting the presence of an amplicon generated during a PCR reaction wherein said PCR reaction comprises

contacting a test sample comprising fungal nucleic acid with appropriate primers in the presence of appropriate nucleotide triphosphates and an agent for polymerisation wherein the detection of said amplicon is directly related to both the presence and absence of a mutation in said nucleic acid wherein the presence of said mutation gives rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene, and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of an amplicon generated during the PCR reaction.

39. A method according to claim 37 or claim 38 comprising contacting a test sample comprising fungal nucleic acid with diagnostic primers to detect both the presence and absence of a specific mutation in said nucleic acid the presence of which gives rise to fungicide resistance to a fungicide whose target protein is encoded by a mitochondrial gene, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primers relating to the absence and the presence of the specific mutation are extended when the appropriate fungal template is present in the sample; and detecting and quantifying the relative presence and absence of the said mutation by reference to the presence or absence of diagnostic primer extension products.

40. A method of selecting an active fungicide and optimal application levels thereof for application to a crop comprising analysing a sample of a fungus capable of infecting said crop and detecting and/or quantifying the presence and/or absence of a mutation in a gene from said fungus wherein the presence of said mutation may give rise to resistance to a fungicide whose target protein is encoded by a mitochondrial gene and then selecting an active fungicide and optimal application levels thereof.

41. A method according to claim 40 wherein the detection method uses any (or a) single nucleotide polymorphism detection technique.

42. A method according to claim 40 or claim 41 wherein the detection method comprises contacting a test sample comprising fungal nucleic acid with a diagnostic primer for

the specific mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended when the said mutation is present in the sample; and detecting the presence or absence of the said mutation by reference to the presence or absence of a diagnostic primer extension product.

43. A method of controlling fungal infection of a crop comprising applying a fungicide to the crop wherein said fungicide is selected according to any of claims 40 to 42.

44. A method according to any of claims 34 to 43 wherein the fungicide is a strobilurin analogue or any other compound in the same cross resistance group.

45. A method for detecting fungal resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a mutation in fungal nucleic acid wherein the presence of said mutation gives rise to resistance to a strobilurin analogue or any other compound in the same cross resistance group said method comprising identifying the presence or absence of a single nucleotide polymorphism occurring at a position in the DNA corresponding to one or more of the bases in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the cytochrome b protein.

46. A method for detecting fungal resistance to a strobilurin analogue according to claim 45 said method comprising identifying the presence or absence of a single nucleotide polymorphism occurring at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the cytochrome b protein.

47. A method according to any of the preceding claims wherein the method of detection and/or quantifying is based on fluorescence detection of diagnostic primer extension products.

48. A method according to any of the preceding claims wherein the method of detection involves the use of the Scorpion™ detection system.

49. A method according to any of claims 34 to 48 wherein said mutation occurs at a position in the DNA corresponding to the second base in the triplet coding for the amino acid at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143 in the cytochrome b protein.

50. A method according to any of claims 34 to 49 wherein the said mutation is a guanine to cytosine change resulting in a G143A replacement in the encoded protein where a wild type glycine residue is substituted with an alanine residue at the position corresponding to *S. cerevisiae* cytochrome *b* residue 143.

51. A computer readable medium having stored thereon any of the sequences in any of the previous claims including all or part of a DNA sequence or protein sequence encoding a mutant cytochrome *b* protein as herein described wherein the presence of a mutation gives rise to fungal resistance to a strobilurin analogue or any compound in the same cross resistance group; all or part of a DNA or protein sequence encoding a wild type cytochrome *b* sequence from a fungus selected from the group *Plasmopara viticola, Erysiphe graminis f.sp. tritici/hordei, Rhynchosporium secalis, Pyrenophora teres, Mycosphaerella graminicola, Venturia inaequalis, Mycosphaerella fijiensis* var. *difformis, Sphaerotheca fuliginea, Uncinula necator, Colletotrichum graminicola, Pythium aphanidermatum, Colletotrichum gloeosporioides, Oidium lycopersicum, Magnaporthe grisea ,Phytophthora infestans, Leveillula taurica, Pseudoperonospora cubensis, Alternaria solani, Rhizoctonia solani, Mycosphaerella musicola and Cercospora arachidola;* or any allele specific oligonucleotide; allele specific primer, allele specific oligonucleotide probe, common or diagnostic primer according to any of the preceding claims..

52. A diagnostic kit for use in a method according to any of claims 1 to 13, 21 or 34 to 50.

53. A diagnostic kit according to claim 52 comprising one or more of the following: diagnostic, wild type, control and/or common oligonucleotide primers, allele specific oligonucleotide probes, appropriate nucleotide triphosphates, for example dATP, dCTP, dGTP, dTTP, a suitable polymerase, and a buffer solution.

Fig.1.

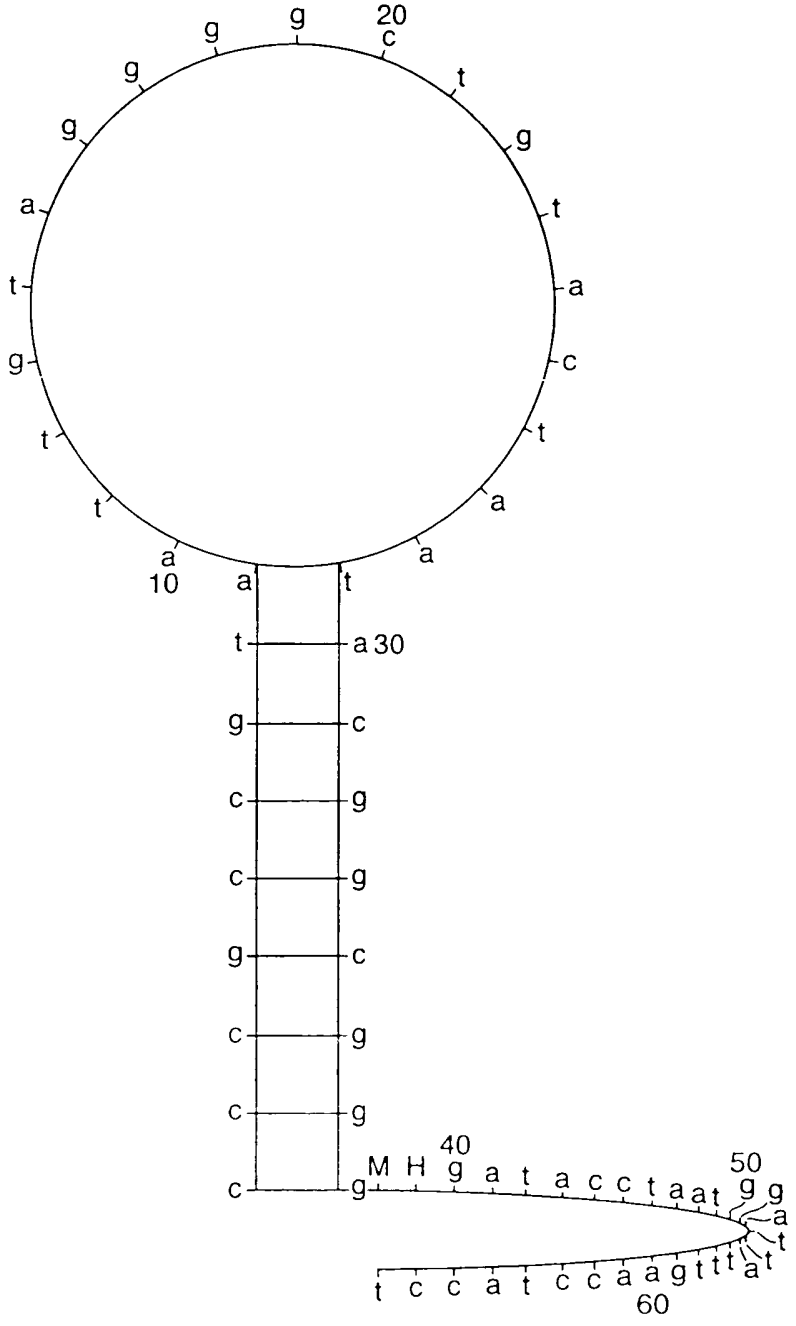


Fig.2a.

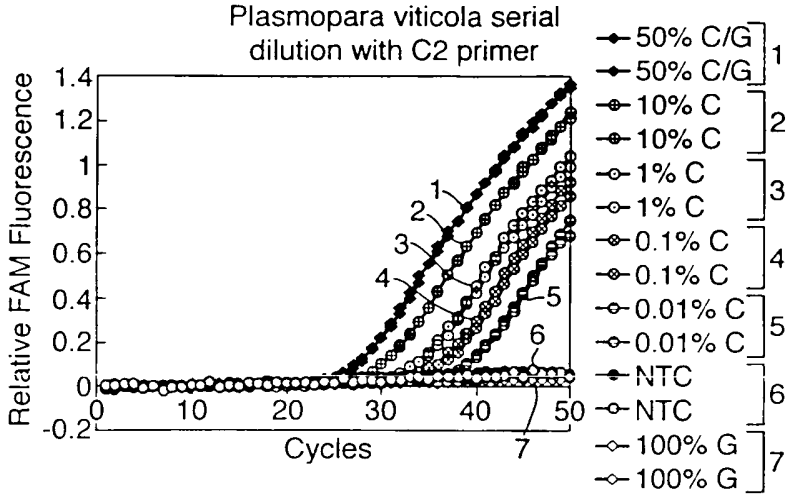

Plasmopara viticola serial dilution with C2 primer
Relative FAM Fluorescence
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
10
20
30
40
50
Cycles
1
2
3
4
5
6
7
50% C/G
50% C/G
10% C
10% C
1% C
1% C
0.1% C
0.1% C
0.01% C
0.01% C
NTC
NTC
100% G
100% G
1
2
3
4
5
6
7

Fig.2b.

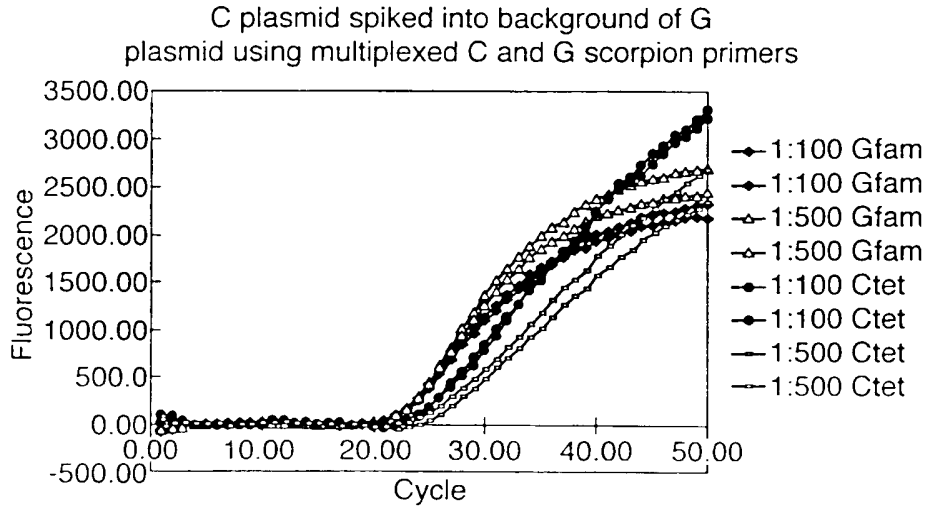
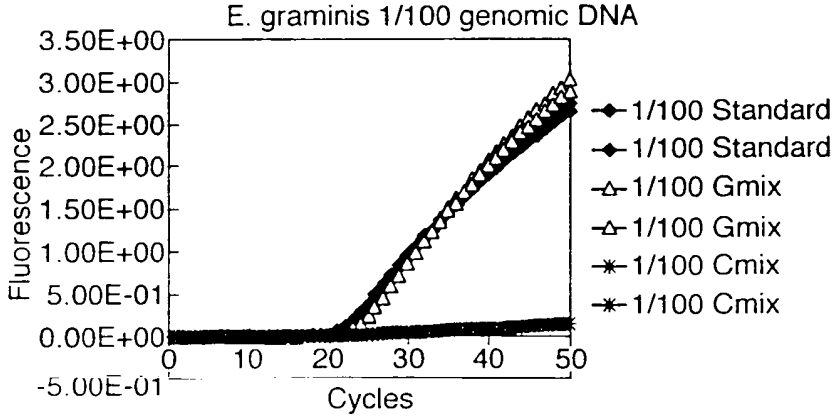

C plasmid spiked into background of G plasmid using multiplexed C and G scorpion primers
Fluorescence
3500.00
3000.00
2500.00
2000.00
1500.00
1000.00
500.0
0.00
-500.00
0.00
10.00
20.00
30.00
40.00
50.00
Cycle
1:100 Gfam
1:100 Gfam
1:500 Gfam
1:500 Gfam
1:100 Ctet
1:100 Ctet
1:500 Ctet
1:500 Ctet

Fig.3a.


E. graminis 1/100 genomic DNA
3.50E+00
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2.50E+00
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
-5.00E-01
Fluorescence
0
10
20
30
40
50
Cycles
1/100 Standard
1/100 Standard
1/100 Gmix
1/100 Gmix
1/100 Cmix
1/100 Cmix

Fig.3b.

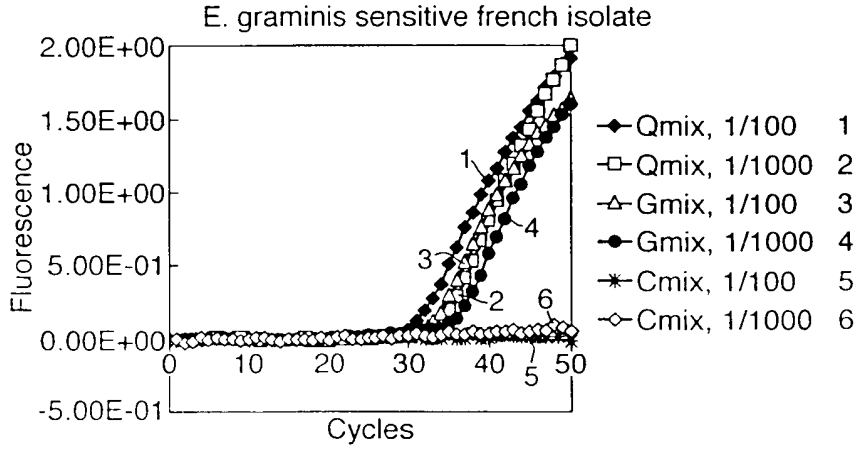
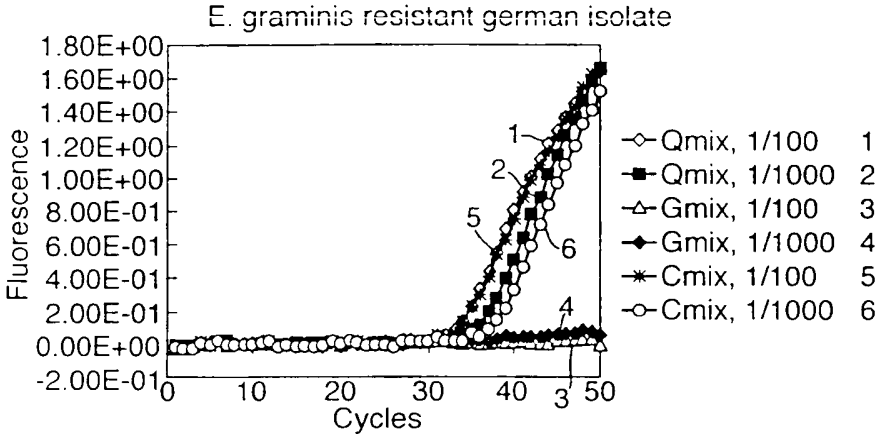

E. graminis sensitive french isolate
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
-5.00E-01
Fluorescence
0
10
20
30
40
50
Cycles
1
2
3
4
5
6
Qmix, 1/100 1
Qmix, 1/1000 2
Gmix, 1/100 3
Gmix, 1/1000 4
Cmix, 1/100 5
Cmix, 1/1000 6

Fig.4.


E. graminis resistant german isolate
Fluorescence
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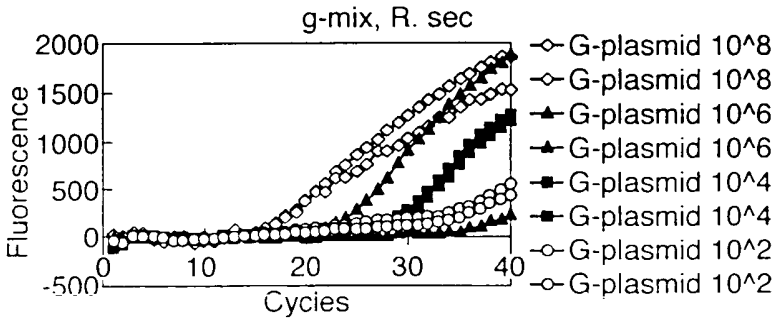
8.00E-01
6.00E-01
4.00E-01
2.00E-01
0.00E+00
-2.00E-01
0
10
20
30
40
50
Cycles
1
2
3
4
5
6
Qmix, 1/100 1
Qmix, 1/1000 2
Gmix, 1/100 3
Gmix, 1/1000 4
Cmix, 1/100 5
Cmix, 1/1000 6

Fig.5a.


g-mix, R. sec
Fluorescence
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1500
1000
500
0
-500
0
10
20
30
40
Cycles
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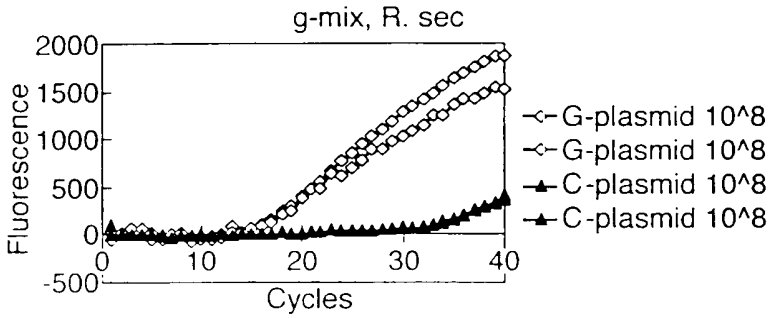
G-plasmid 10^8
G-plasmid 10^6
G-plasmid 10^6
G-plasmid 10^4
G-plasmid 10^4
G-plasmid 10^2
G-plasmid 10^2

Fig.5b.


g-mix, R. sec
Fluorescence
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1500
1000
500
0
-500
0
10
20
30
40
Cycles
G-plasmid 10^8
G-plasmid 10^8
C-plasmid 10^8
C-plasmid 10^8

Fig.6a.

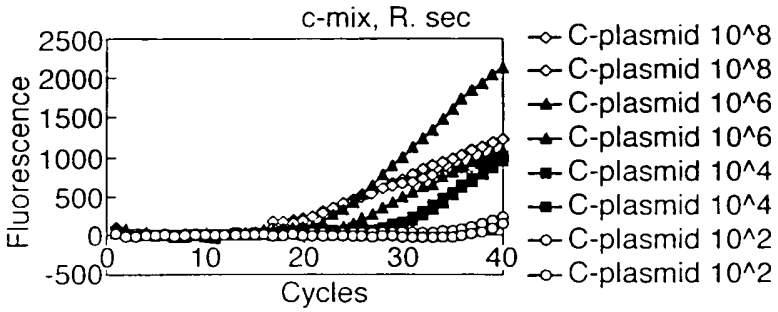
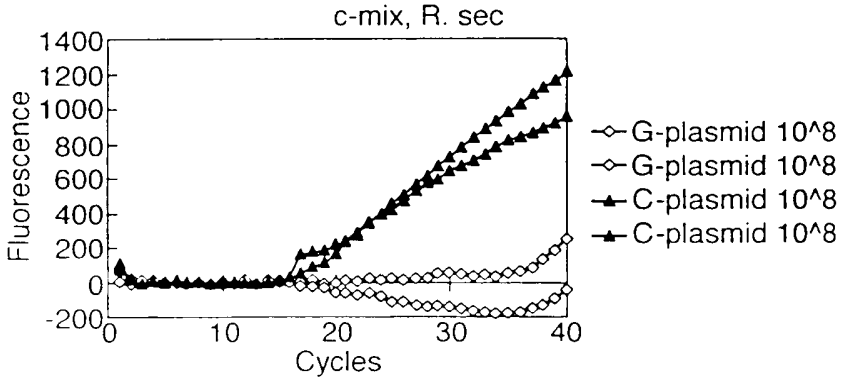

c-mix, R. sec
Fluorescence
2500
2000
1500
1000
500
0
-500
0
10
20
30
40
Cycles
C-plasmid 10^8
C-plasmid 10^8
C-plasmid 10^6
C-plasmid 10^6
C-plasmid 10^4
C-plasmid 10^4
C-plasmid 10^2
C-plasmid 10^2

Fig.6b.


c-mix, R. sec
Fluorescence
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1200
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800
600
400
200
0
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10
20
30
40
Cycles
G-plasmid 10^8
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C-plasmid 10^8
C-plasmid 10^8

Fig.7a.

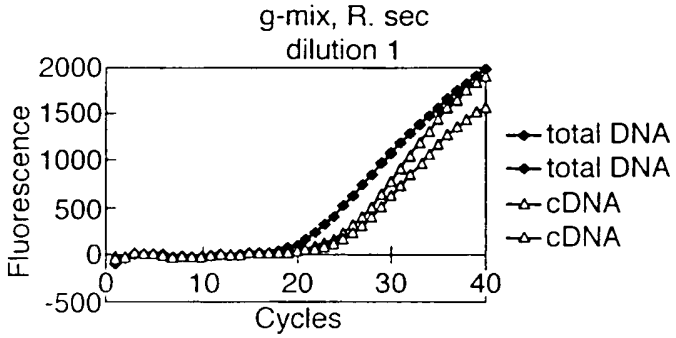


Fig.7b.

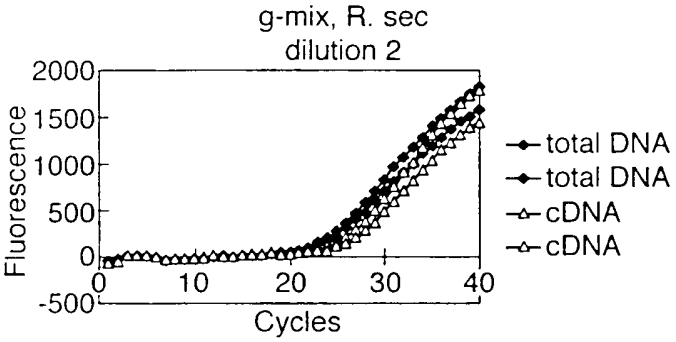


Fig.7c.

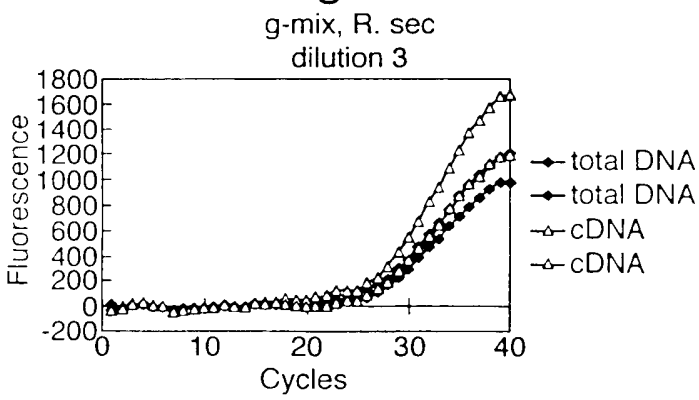


Fig.8a.

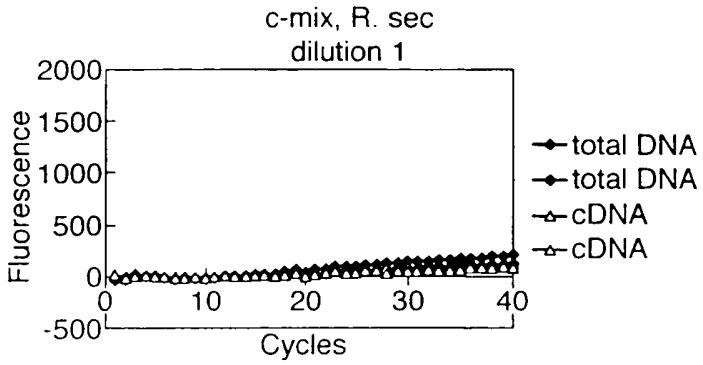


Fig.8b.

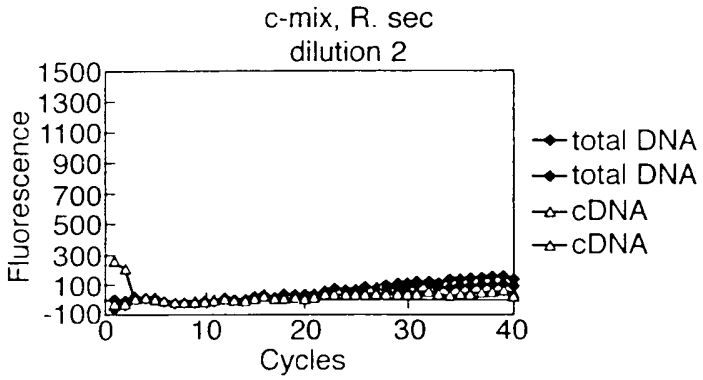


Fig.8c.

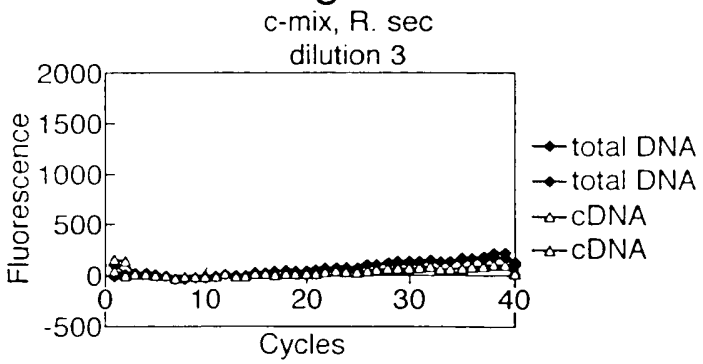
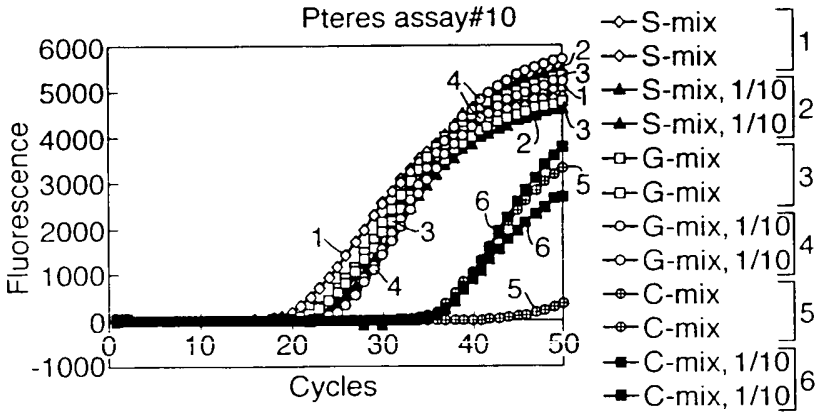


Fig.9a.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4
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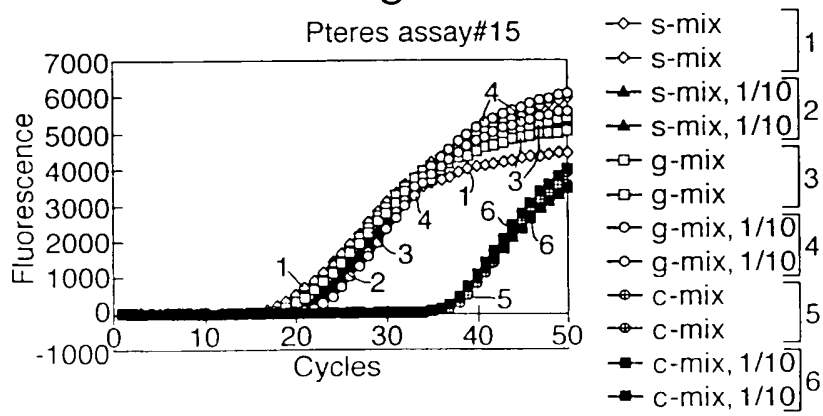
6

Fig.9b.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SEQUENCE LISTING

<110> Zeneca Ltd.
Little, Stephen
Whitcombe, David M
Theaker, Jane
Gibson, Neil J
Stanger, Carole P
Renwick, Annabel
Heaney, Stephen P
Windass, John D

<120> Methods

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(57) Abstract: The invention discloses methods which are particularly sensitive for detecting low frequencies of mutations in mitochondrially encoded genes, such as the cytochrome b gene, making these an especially useful and commercially important way of screening plant pathogenic fungi for the onset of fungicidal resistance wherein the resistance is due to a mutation in a mitochondrially encoded gene. The methods disclosed include single nucleotide polymorphism detection techniques especially PCR detection methods. Also disclosed are DNA sequences encoding part of the wild type and mutant cytochrome b sequences of a range of plant pathogenic fungi and the use of the sequence information to detect mutations giving rise to fungicidal resistance. Allele-specific oligonucleotides and oligonucleotide probes, diagnostic primers and diagnostic kits are also disclosed.

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 C07K14/37 C12N15/53 G06F19/00 G06F17/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C07K C12N G06F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZHENG D ET AL: "Characterization of mitochondrial cytochrome b gene from Venturia inequalis" CURRENT GENETICS, vol. 32, November 1997 (1997-11), pages 361-66, XP000990849 the whole document ---	1-53
Y	DI RAGO J-P AT AL: "Molecular basis for resistance to Myxothiazol, Mucidin (Strobilurin A), and stigmatellin" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 264, no. 24, August 1989 (1989-08), pages 14543-548, XP002164632 the whole document ---	1-53
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5 April 2001	20/04/2001
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040 Tx. 31 651 epo nl Fax: (+31-70) 340-3016	Authorized officer [illegible]

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with [redacted]on, where appropriate, of the relevant passages	Relevant to claim No
Y	KRAICZY P ET AL: "The molecular basis for the natural resistance of the cytochrome bc1 complex from strobilurin-producing basidomycetes to centre Qp inhibitors" EUROPEAN JOURNAL OF BIOCHEMISTRY , vol. 235, 1996, pages 54-63, XP000990816 the whole document ---	1-53
Y	STEFFENS JJ ET AL: "Mechanisms of fungicide resistance in phytopathogenic fungi" CURRENT OPINION IN BIOCHEMISTRY, vol. 7, 1996, pages 348-55, XP000990845 page 348 page 350, paragraph 6 - paragraph 8 ---	1-53
A	CORRAN AJ ET AL: "Approaches ti in-vitro Lead Generation for fungicide invention" PESTICIDE SCIENCE, vol. 54, 1998, pages 338-344, XP002164633 page 339, paragraph 4 - paragraph 7 page 342 ---	1-53
A	NEWTON C R ET AL: "ANALYSIS OF ANY POINT MUTATION IN DNA. THE AMPLIFICATION REFRACTORY MUTATION SYSTEM (ARMS)" NUCLEIC ACIDS RESEARCH,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 17, no. 7, 11 April 1989 (1989-04-11), pages 2503-2516, XP000141596 ISSN: 0305-1048 -----	